METABOLISM AND EXCRETION OF PHENOTHIAZINE TRANQUILLISERS BY THE HORSE

A THESIS PRESENTED TO THE UNIVERSITY OF GLASGOW FOR THE DEGREE OF Ph.D.

by

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C O N T E N T S.

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ABSTRACT

The historical development, uses, pharmacology, chemistry and metabolism of phenothiazine and the phenothiazine tranquillisers are reviewed along with the analytical techniques which have been used for their detection and determination.

The metabolism and excretion by the horse of promazine chlorpromazine, acepromazine and propionylpromazine, tranquillisers commonly used in equine practice, have been examined qualitatively and quantitatively. Methods of collection, storage and analysis of horse urine have been developed, and the use of mass spectroscopy in combination with gas liquid chromatography for the detection of phenothiazine derivatives in biological fluids has been examined.

Qualitative results have shown that many biotransformations of such derivatives take place in the horse. 30 metabolites of promazine were detected in addition to the parent drug, 20 of chlorpromazine and 11 and 6 respectively from acepromazine and propionylpromazine. Hydroxylation followed by conjugation, predominantly with glucuronic acid and to a smaller extent with sulphuric acid, is the major route of metabolism of promazine and chlorpromazine. Acepromazine and propionylpromazine, on the other hand, are not conjugated to a great extent. Instead they lose the side chain ketone grouping attached to the nucleus at the 2-position and are excreted in the sulphoxide form.

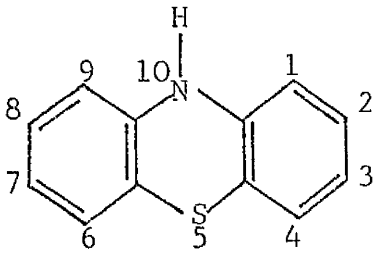
Quantitative results have shown that excretion is irregular and prolonged, in some cases lasting for a week after administration. The percentage of dose detected as urinary metabolites was low,

being approximately 19% for chlorpromazine, 10% for promazine and 3% for acepromazine. Conjugated metabolites were excreted predominantly as sulphide derivatives, whereas the unconjugated fraction were predominantly in the sulphoxide form.

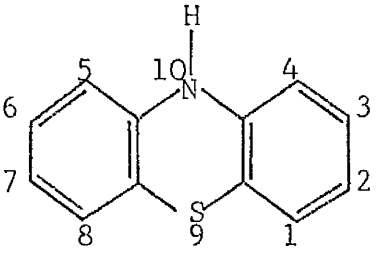
The applicability of the techniques used for the detection of phenothiazine derivatives, after administration in small doses just sufficent to produce an effect, has also been examined.

NOMENCLATURE

Three different numbering systems have been used by different authors at different times to designate the positions on the phenothiazine ring system. The first (I) is that advocated by the International Union of Pure and Applied Chemistry, the Chemical



(I)



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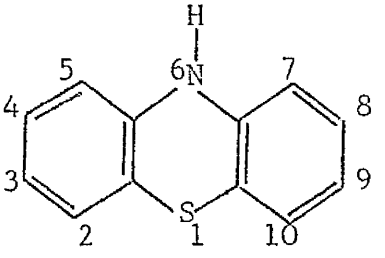
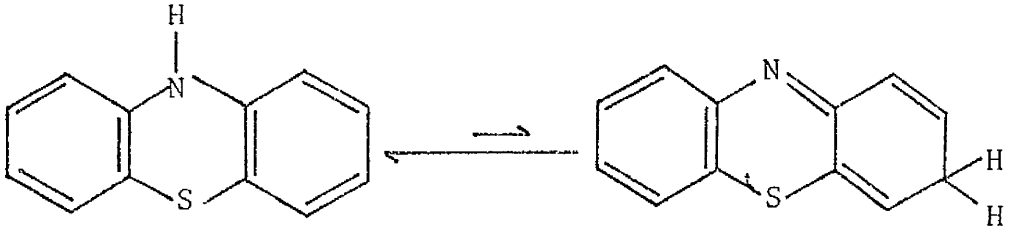


(III)

Society and Chemical Abstracts. The second system (II) appears in Beilstein's "Handbuch der organischen Chemie", 4th edition, and system (III) may be found in Meyer-Jacobson's "Lehrbuch der organische Chemie" and F.F. Richter's "Lexikon der Kohlenstoffverbindungen".

The system of nomenclature used in the present study is that accepted by the International Union of Pure and Applied Chemistry (I). In addition, throughout the text the term "phenothiazine" refers to



(A) (B)

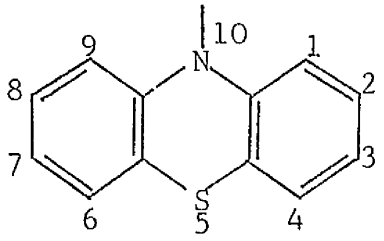
10H - phenothiazine (A), the hypothetical isomer 3H-phenothiazine (B) being referred to as such when necessary.

HISTORICAL BACKGROUND, PHARMACOLOGICAL PROPERTIES AND CLINICAL USES OF THE PHENOTHIAZINE TRANQUILLISERS.

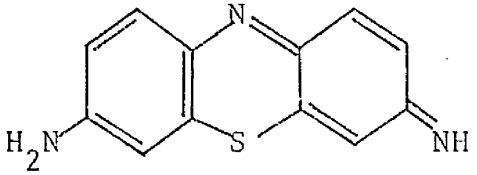
I. HISTORICAL BACKGROUND, PHARMACOLOGICAL PROPERTIES AND CLINICAL USES OF THE PHENOTHIAZINE TRANQUILLISERS

A. Development of Phenothiazine.

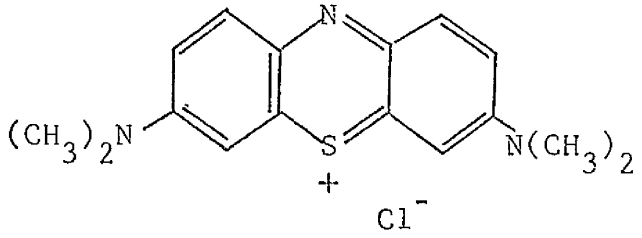
Phenothiazine, the chemical nucleus of the modern 'phenothiazine' tranquillisers, was first prepared by Bernthsen (1883) during studies into the structures of the two dyes Lauth's Violet, (7-amino-3-imino-3H-phenothiazine hydrochloride- Lauth, 1876), and methylene blue, (3, 7-bis (dimethylamino)-phenazothionium chloride - Caro, 1876).



Phenothiazine



Lauth's Violet



Methylene Blue

In initial experiments he achieved the synthesis by fusion of sulphur and diphenylamine at 250-260°C, and went on to prove its structure in a series of sophisticated studies (Bernthsen, 1883). The same method of synthesis is employed to the present day, although a few refinements have been added. In particular, iodine (Ackermann, 1911a; Knoevenagel, 1914) or aluminium chloride (Ackermann 1911b) has been used as a catalyst, and a purer product has been obtained using a carbon dioxide atmosphere (Geiger and Beck, 1948).

Although seldom used as the starting material for phenothiazine dyes, its role as the nucleus of such compounds has proved of great interest to researchers in the dyestuffs industry (Venkatamaran, 1952). It has also been used industrially as an antioxidant for petrochemicals, showing a high degree of inhibition (Murphy, Rainer and Smith, 1950; Smith, 1951).

The compound was of little commercial interest until its insecticidal properties were discovered by Campbell, Sullivan, Smith and Haller (1934), who demonstrated its lethal action on the larvae of culicine mosquitoes. The extensive investigations which followed showed that phenothiazine has toxic effects on a large range of economically important insects (Zukel, 1944). This worker attributed its insecticidal properties to cytochrome oxidase inhibition by an unspecified conjugate of leucothionol (3, 7 - dihydroxy phenothiazine). However, reports of photo-sensitization of human skin by phenothiazine dust, or sprays, restricted its widespread use in this field (Harwood, 1953).

The anthelmintic properties of phenothiazine were first discovered by Harwood, Jerstad and Swanson (1938), who investigated its effects on ascarids and nodular worms in swine. It has since been tested on nematode parasites in most domestic animals and man (Davey and Innes, 1942). A particular advantage of phenothiazine, which had not been observed with other anthelmintics, was its ability to control worm levels by reducing egg production,

when administered in repeated doses too small to have a lethal effect on the parasite (Findlay, 1950).

It is thought to enter the parasite through the cuticle (Lazarus and Rogers, 1950, 1951), and many theories have been put forward regarding the active form (Collier, 1940; De Eds and Thomas, 1941). Phenothiazine sulphoxide, (Phenothiazine-5-oxide), has been reported to possess anthelmintic activity, whereas the sulphone, (phenothiazine-5,5-dioxide), was inactive (Rogers, Cymerman- Craig and Warwick, 1955). Similarly, phenothiazone, (3H-phenothiazine-3-one), and thionol, (3-hydroxy-3H-phenothiazine-7-one), showed no activity (Gordon and Lipson, 1940; Taylor and Sanderson, 1940; Rogers et al., 1955). On this basis Craig and Tate (1961) suggested that the sulphoxide, formed *in vitro* at the site of action, is the active species. This is in keeping with the findings of Clare (1947), who reported that sulphoxidation of phenothiazine occurs in the rumen of sheep and cattle. This was later confirmed by Harpur, Swales and Denstedt (1950). The subject has been discussed in detail by Davey and Innes (1942) and Craig and Tate (1961).

Although at first considered non-toxic, several reports of haemolytic anaemia after phenothiazine administration put an end to its use as an anthelmintic in humans (Findlay, 1950; Harwood, 1953). However, the compound is still extensively used in veterinary medicine (Griffiths, 1954). Its anthelmintic uses have been extensively reviewed by Davey and Innes (1942), In addi

(1947), Findlay (1950), Harwood (1953), Griffiths (1954), and Craig and Tate (1961).

Many other medical uses of phenothiazine have been reported over the last 30 years. It acts as a urinary antiseptic (De Eds, Stockton and Thomas, 1939; De Eds and Thomas, 1942), a tuberculostatic agent (Freedlander, 1944), and its derivatives have been used as antiemetics (Friend and Cummins, 1953), antihistamines (Halpern, 1946; Halpern and Ducrot, 1946), and in the treatment of Parkinson's disease (Burger, 1951). During the last two decades interest in substituted phenothiazines, especially the 10-substituted derivatives, has increased enormously due to their unique tranquillising properties.

B. Development of the 'Phenothiazine' Tranquillisers.

Since certain phenothiazine derivatives, (e.g. methylene blue), had earlier shown antimalarial activity (Guttman and Ehrlich, 1892), and since phenothiazine itself had been found useful as an anthelmintic, Gilman and Shirley (1944) synthesised a number of substituted phenothiazines in a search for more effective agents. Included amongst these were a series of 10-dialkylaminoalkyl derivatives. However, they were found to be lacking in such activity and the investigation was closed.

At the same time a similar 10-substituted series was synthesised independently in France, and , in addition to antimalarial and anthelmintic screening, was tested for antihistaminic activity. This resulted in the discovery of considerable antihistaminic activity in two compounds, 10-(2-dimethylaminoethyl)-

phenothiazine, (RP3015), and 10 -(2-dimethylamino - 1 - propyl)-phenothiazine, (Promethazine) (Halpern, 1946; Halpern and Ducrot, 1946). They were also found to be less toxic than any previously reported antihistamines (Halpern and Cruchaud, 1947), and to have sedative and local anaesthetic properties (Halpern, Perrin and Dews, 1947).

The discovery of biological activity in 10-substituted phenothiazines led immediately to the synthesis of a large range of such compounds, many of which were found to be pharmacologically active (Charpentier, 1947; Charpentier and Ducrot, 1951; Charpentier, Gailliot and Gaudechon, 1951; Charpentier, Gailliot, Jacob, Gaudechon and Buisson, 1952).

Amongst the first derivatives studied were 10-(2-diethyl-aminoethyl)phenothiazine, (Diethazine), and 10-(2-diethylamino -1-propyl)-phenothiazine, (Ethopropazine), which in addition to exhibiting antihistaminic properties (Bovet, Fournel and Charpentier, 1947; Schaepdryver, 1950) were also effective in the treatment of Parkinson's disease (Bovet, Durel and Longo, 1950; Burger, 1951). 10-(3-dimethylamino -1- propyl)-phenothiazine, (Promazine), was also investigated, but although effective as a local anaesthetic, it showed little antihistaminic activity (Halpern, Perrin and Dews, 1947). Its value as an ataractic was not realised until much later. (Begany, Seifter and Pless, 1956).

The action of such compounds on the central nervous system

was later reported by Laborit and co-workers, who demonstrated their potentiating action in general anaesthesia. In initial studies promethazine or diethazine was used in combination with the anaesthetic, but later investigations showed that 2-chloro-10-(3-dimethylamino -1- propyl)-phenothiazine, (Chlorpromazine), was the derivative of choice (Laborit, Huguenard and Alluaume, 1952; Laborit and Huguenard, 1954). The latter compound, synthesised by Charpentier et al, (1952), was also found to possess a wide range of pharmacological properties (Courvoisier et al, 1952). The most important of these was its ability to calm mentally disturbed patients (Delay, Deniker and Hart, 1952), and it has since been extensively used in the psychiatric field.

This discovery prompted further synthesis and research into the pharmacological properties of such derivatives which resulted in the large series of 10-substituted phenothiazines known collectively as the phenothiazine tranquillisers. They have since been extensively used in psychiatric wards in the management and treatment of psychoses and neuroses, sedation being achieved without the loss of consciousness, or the drowsiness, usually associated with barbiturates and other hypnotics. A representative series of tranquillisers is shown in Table 1.

C. Pharmacology.

The group has a complex pharmacology, acting on both the central and autonomic nervous systems. On the former they have

sedative effects and diminish conditioned avoidance behaviour. They possess antiemetic and antipruritic activity, and have a degree of potency as analgesics. Endocrine changes and seizure discharge can also be effected, along with changes in skeletal muscle tone, and temperature regulation.

They have antihistaminic and antiserotonin activity and, on the autonomic system, act as adrenergic and cholinergic blocking agents. They potentiate the action of various narcotic drugs, and have a degree of potency as local anaesthetics. They also have a large number of undesirable side effects including extra-pyramidal reactions (e.g. parkinsonian tremors), hypersensitivity reactions (e.g. photosensitivity), agranulocytosis, jaundice, and dermatitis.

Although as a group they possess many similar pharmacological properties, the degree of activity and of adverse side effects varies considerably from compound to compound, and from species to species. The pharmacology of these drugs in humans has been reviewed in detail by Courvoisier et al. (1952) and Domino (1962).

D. Structure-Activity Relationships.

The activity of such derivatives has been loosely related to structure, the determining factors being the substituents at the 2- and 10- positions. Substitution at the 2-position renders the molecule asymmetrical, and has been found to increase its activity (Goodman and Gilman, 1965). Thus in the 'promazine'

series (Table 1) substitution of a methoxy, acetyl, or trifluoromethyl group, or of a chlorine atom, results in increased potency to depress the central nervous system, (c.f. promazine, methoxypromazine, acepromazine, chlorpromazine, triflupromazine). In addition, compounds with a trifluoromethyl group tend to produce reactions of an extrapyramidal nature, (triflupromazine, trifluoperazine, fluphenazine).

The activity of compounds with a three carbon polymethylene chain between the two nitrogen atoms seems to increase according to the 2-substituent in the order unsubstituted = methoxy < acetyl< chloro < trifluoromethyl (Carey and Sanford, 1963). On the other hand the activity of compounds with a two carbon polymethylene chain tends to decrease with substitution at the 2-position, (c.f. promethazine, chlorpromethazine, propiomazine).

The potency of such compounds can also be broadly divided into three groups depending on the nature of the side chain at the 10-position. The least active derivatives possess an aliphatic side chain terminating in a dialkylamino group, (promazine, chlorpromazine, acepromazine, triflupromazine). With these compounds tranquillisation is usually accompanied by a degree of sedation. The nature of the aliphatic chain also determines to some extent the specific activity of the compound. Thus those with a three carbon chain have greater central depressant and antiemetic activity, ('Promazine' series), whereas a two carbon chain tends to produce antihistaminic activity

and anti-Parkinsonism effects, (promethazine, pyrathiazine; diethazi ethopropazine).

The second group has approximately the same central depressant activity as the first, in this case the terminal side chain amino group being replaced by a substituted piperidine ring. Such compounds include mepazine and thioridazine.

The remaining group, with much greater central activity, incorporates a substituted piperazine ring in place of the terminal nitrogen atom and includes the 'perazine' series, the 'phenazine' series and thiopropazate (Table 1). In this case tranquillisation is achieved without appreciable sedation. They have a large degree of antiemetic activity, and extrapyramidal reactions are common.

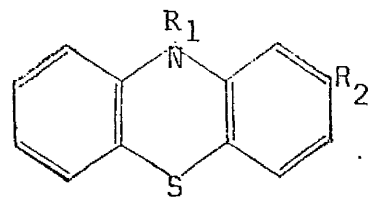
Structure-activity relationships between the phenothiazine tranquillisers have been reviewed and discussed by Friend (1960).

E. Classification.

Many suggestions have been put forward as to the classification of phenothiazine derivatives in the broader field of tranquillising and sedative drugs. There is difficulty in classification since, on the one hand, small changes in structure within a chemically related group can give rise to vast differences in pharmacological activity, (chlorpromazine and promethazine), whereas, on the other hand, compounds completely unrelated chemically can produce the same therapeutic action, (chlorpromazine and reserpine).

Berger (1957) suggested a classification based both on their

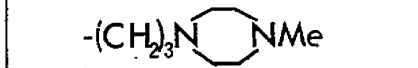
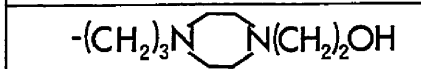
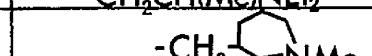
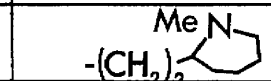
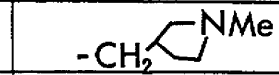
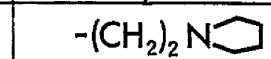
Table 1. CLASSIFICATION OF THE PHENOTHIAZINE TRANQUILLISERS ON THE BASIS OF CHEMICAL STRUCTURE. (See next page)



General formula

* Denotes Major Use

A = Tranquilliser
B = Antihistamine
C = Antiemetic
D = Antipruritic
E = Anti-Parkinsonism

SUBGROUP ON NITROGEN	R_1	EXAMPLES	R_2	TRADE NAME	USE*
Dimethylaminopropyl	$-(CH_2)_3NMe_2$ 'Promazine' group	Acetylpromazine	-COMe	Notensil	A
		Chlorpromazine	-Cl	Largactil	AC
		Methoxypromazine	-OMe	Mopazine	A
		Promazine	-H	Sparine	A
		Triflupromazine	$-CF_3$	Vespral	A
	$-CH_2CH(Me)CH_2NMe_2$	Methotrimeprazine	-OMe	Nozinan	A
		Trimeprazine	-H	Vallergan	D
Propyl piperazine	 'Perazine' group	Prochlorperazine	-Cl	Stemetil	A
		Thiethylperazine	-SEt	Torecan	C
		Thioperazine	$-SO_2NMe_2$	Majeptil	AC
		Trifluoperazine	$-CF_3$	Stelazine	A
	 'Phenazine' group	Acetophenazine	-COMe	Tindal	A
		Carphenazine	-COEt	Proketazine	AC
		Fluphenazine	$-CF_3$	Prolixin	A
		Perphenazine	-Cl	Fentazin	A
		Thiopropazate	-Cl	Dartal	A
Dimethylaminoethyl	$-CH_2CH(Me)NMe_2$ 'Promethazine' group	Chlorpromethazine	-Cl	—	B
		Promethazine	-H	Phenergan	B
		Propiomazine	-COEt	Propiomazine	B
Diethylaminoethyl	$-(CH_2)_2NEt_2$	Diethazine	-H	Diparcol	E
	$-CH_2CH(Me)NEt_2$	Ethopropazine	-H	Parsitan	E
Methylpiperidyl		Mepazine	-H	Pacatal	A
Ethylpiperidyl		Thioridazine	-SMe	Mellaril	A
Propylpiperidyl		Pipamazine	-Cl	Mornidine	C
Methylpyrrolidyl		Methdilazine	-H	Tacaryl	B
Ethylpyrrolidyl		Pyrathiazine	-H	Pyrrolazote	B

chemical structure and action on the nervous system. The phenothiazines and the Rauwolfia alkaloids were classed as tranquillisers with effects on both central and autonomic systems, whereas the propanediols and diphenylmethane derivatives were classified as only affecting the central nervous system.

Several authors have suggested classification of tranquillisers on the basis of their specific use in psychiatry. (Schiele and Benson, 1958; Ross and Cole, 1960). In this system they were grouped as having either major or minor activity. The major group were those used to control patients with more severe mental disorders, which may produce extra-pyramidal reactions, but do not induce physical dependence. The minor group were those used in the treatment of slight neuroses and psychoses, and of psychosomatic disorders. Again this grouping is very general, and the two classes overlap considerably.

A similar classification was put forward by a study group of the World Health Organisation. (W.H.O., 1958), dividing tranquillisers and sedatives into three groups. The first group, the major tranquillisers, comprised those used in more severe psychiatric disorders and included the stronger phenothiazine tranquillisers such as chlorpromazine and perphenazine, the Rauwolfia alkaloids, butyrophenones such as haloperidol, and thioxanthene derivatives such as chlorprothixene.

The minor tranquillisers were those used in the treatment

of anxiety, psychosomatic disorders, nervous tension, and the milder neuroses and psychoses. Included were the weaker phenothiazine tranquillisers such as promazine and promethazine, diphenylmethane derivatives such as hydroxyzine and azacyclonol, and chlordiazepoxide. A third group, based on their much more marked sedative properties included the dithiocarbamates , such as meprobamate, and phenaglycodol.

There can be no absolute classification of tranquillising and sedative agents on the basis of specific therapeutic activity due to the wide range of pharmacological properties exhibited by those drugs. However the methods of classification described give some insight into the position of phenothiazine tranquillisers in the broader spectrum of tranquillising and sedative agents. The problems of such classification have been discussed in detail by Domino (1962).

F. Tranquillisers in Veterinary Medicine.

Apart from their use in general medicine and psychiatry, these compounds have proved extremely useful in the veterinary field in the restraint, management and treatment of temperamental animals, and as premedication for general anaesthesia. Although there have been many reports of undesirable side effects from these drugs in animals, the toxicity is appreciably less than in man. (Dubost and Pascal, 1953; Dobkin, Gilbert and Lamoureux, 1954).

They have a sedative effect in all species, the degree depending on the specific drug, dose and species, and also on the individual animal. Typically the animal develops a relaxed posture and shows no sign of anxiety. It does not appear to be aware of its surroundings, but can be aroused if stimulated. This depression is usually most marked over the first six hours after administration, but residual signs are often detected up to 48 hours. With smaller doses the animal tends to become sleepy, and from E.E.G. studies, this state seems to resemble natural sleep rather than the drowsiness produced by barbiturates, (Westhues and Fritsch, 1965). When higher doses are employed muscular weakness occurs and the animal tends to be unsteady when standing, or may lie down. Occasionally, with certain drugs, especially when administered to horses, an abnormal excitement reaction takes place (Pg. 25).

Other visible signs associated with tranquilliser therapy are enophthalmus, miosis, ptosis and protrusion of the third eyelid. In male animals the penis is relaxed and protrudes from the sheath. The drugs also produce hypothermia and can cause hypotension and irregular respiration and pulse rates. Although all the above reactions have been noted after administration of tranquillisers to animals, effects and visible signs vary for individual compounds and from species to species.

The most important clinical applications of these

compounds are basically the same in all species, and will be discussed with emphasis on their use in equine practice (Pg. 24)

In particular, chlorpromazine, promazine and acepromazine have been widely used in the veterinary field. Chlorpromazine has a marked sedative action and adrenolytic activity. It is a powerful antiemetic and a potent vagolytic agent. It is commonly used in veterinary medicine as premedication for general anaesthesia and chlorpromazine therapy has been extensively described in the treatment of domestic animals (Troughton, Gould and Anderson, 1955; Brodey and Thorald-Christensen, 1956; Estrada, 1956; Weaver, 1956; Martin and Beck, 1956; Clifford, 1957; Graves, 1957).

Promazine is closely related to chlorpromazine and has somewhat similar actions, although sedation is not so marked and is of shorter duration. Its clinical and surgical uses have been reported in detail in small animals, (Gradess, 1956; Clifford, 1957; Knowles, 1957; Krawitz, 1957), swine. (Nelson, 1958), cattle (Schulz, 1958) and horses (Owen and Neal, 1957; Shambaugh, 1958; Dawson, Lickfeldt and Brengle, 1959; Gorman, 1959; Raker and English, 1959; Raker and Sayers, 1959).

Acepromazine, one of the more recently discovered tranquillisers (Schmitt et al., 1957) has properties similar to promazine, but has the advantage of requiring very small doses. Although references to it in British Veterinary literature are few (Cooper, 1961; Van Laun, Nettleton and Japp, 1961; Baillie, 1967), it has been extensively used abroad as a tranquilliser

and preanaesthetic agent in domestic animals. In particular, its usefulness has been widely reported in Scandinavia (Hansen, 1958; Korsbach, 1958; Nordstrom, Orstradius and Lanek, 1958; Ostradius and Lanek, 1960; Sonnichsen, 1960).

The other phenothiazine derivatives used in veterinary medicine have properties similar to the three compounds mentioned, but their application in this field has been more limited.

G. Tranquillisers in Equine Practice.

These drugs have proved exceptionally useful in equine practice, both due to the size and temperament of members of this species. They are used as sedatives in the examination and treatment of nervous, aggressive, or excitable animals, especially when surgery under local anaesthesia is involved. This sedative effect is also used to reduce the apprehension of such animals during loading or transport, or when placed in strange surroundings.

They have proved invaluable as premedicants for general anaesthesia, reducing excitement during induction and recovery, and potentiating the action of the anaesthetic. Such compounds have also been used in the treatment of shock (Westhues and Fritsch, 1965), tetanus (Owen, 1955; Troughton, Gould and Anderson, 1955; Shambaugh, 1958; Dawson, Lickfeldt and Brengle, 1959) and colic (Troughton, Gould and Anderson, 1955).

Variable effects are produced after their administration to

the horse, which can be classified broadly into three groups. The first type is the normal tranquillisation experienced in most species. In the horse this takes the form of lowering of the head, immobility, and a sleepy appearance, with drooping eyelids, ears or mouth. Occasionally nasal discharge, salivation, shivering and sweating are observed. In the case of male animals, as in all species, the penis becomes relaxed and protrudes from the sheath. The animal appears to show no interest in its surroundings, and is not readily aroused.

In the second case there seems to be complete lack of response to the drug. After administration the animal stands quietly, but does not have the sedated appearance noted in the first type of reaction. This effect, for reasons unknown, seems to be due to the inability of the drug to function normally (Carey and Sanford, 1963). Such findings have been reported by several workers (Martin and Beck, 1956; Owen and Neal, 1957; Dawson, Lickfeldt and Brengle, 1959; Gorman, 1959; Cosgrove, Collins, Ravenagh and Cosgrove, 1960).

Occasionally, especially with thoroughbred horses, a third type of reaction occurs, being a complete reversal of the normal action of the drug. It has most commonly been observed after administration of chlorpromazine, although it has been noted from time to time with promazine, acepromazine, propionylpromazine, trimeprazine, or perphenazine (Owen and Neal, 1957; Williams and Young, 1958; Nordstrom, Orstradius and Lanek, 1958; Gorman,

1959; Jones, 1963; Westhues and Fritsch, 1965). At first the animal stands quietly, but after a few minutes becomes excited, uneasy and unsteady. It will sink back on its haunches, and may rear, neigh, and paw the ground, besides plunging around with uncoordinated movements. This condition recurs at periods of several hours alternating with periods of sedation and has been found to be associated with an increase in pulse rate (Gorman, 1959; Hall, 1960; Jones, 1963; Westhues and Fritsch, 1965). This type of uncoordinated excitement has also been noted in dogs and pigs (Westhues and Fritsch, 1965). Jones (1963) succeeded in reversing the reaction in a horse by means of an intravenous injection of methylamphetamine.

Several theories have been put forward as to the cause of this third reaction. One suggestion is that the effect is caused by hypotension giving rise to vertigo (Owen and Neal, 1957). However, investigations of this theory, after administration of chlorpromazine, have shown no fall in blood pressure (Hall, 1960; Carey and Sanford, 1963). Hall, on the other hand, found a slight increase in blood pressure, and a large increase in pulse rate when the condition was evident. Bente (1957) suggested that the effect is due to the alternating dominance of opposing depressant and excitant reactions of the autonomic nervous system, whereas Owen and Neal (1957) suggested that it is a panic reaction in the animal due to a sensation of muscular weakness. These theories, however, have not been substantiated.

Side effects of phenothiazines have also been investigated

in the horse. Hypotension does not appear to occur to any appreciable extent. Martin and Beck (1956) noted a slight fall in blood pressure after intramuscular administration of chlorpromazine ($\sim$ 2mg/kg). On the other hand Carey and Sanford (1963) detected no change, and Hall (1960) reported a slight hypertension with this drug (0.5 mg/kg). Rapid intravenous injection of promethazine has been found to produce a distinct fall in blood pressure (Hall, 1960). A very slight hypotension was noticed after administration of chlorpromazine (2-4mg/Kg), although with larger doses a greater drop was observed (Martin and Beck, 1956; Owen and Neal, 1957). A fall of $3F^{o}$ was reported by Alexander (1960) two hours after administration of chlorpromazine (1.5 mg/kg.) returning to normal after seven hours. After intravenous administration of promazine ($\sim$ 1.5 mg/Kg) Carey and Sanford (1963) reported a fall in rectal temperature of $5F^{o}$ within two hours.

Under normal conditions of sedation, the effect on pulse rate is slight, an increase usually being produced. A slight tachycardia has been reported after administration of chlorpromazine (Martin and Beck, 1956; Hall, 1960; Jones, 1963), this being greatly accentuated during the excitement reaction (Gorman, 1959; Hall, 1960; Jones, 1963; Westhues and Fritsch, 1965). A noticeable increase in pulse rate has also been reported following administration of large doses of acepromazine (Sonnichsen, 1960).

Effects on pulse rate after administration of promazine are variable. Carey and Sanford (1963) found that the rate was virtually unaltered after doses of 0.5-1 mg/kg. Raker and English (1959) administered repeated intravenous doses (~1mg/Kg) to two horses every three days for two weeks. After each dose they found the pulse rate had dropped by 10 minutes after injection, but had risen to a maximum by 45 minutes, gradually returning to normal within three hours. The decrease was of the order of 10-20% and the subsequent increase from 30-50%. However, with other horses they found only slight increases.

The duration of action of these drugs has also been found to vary with dose, route of administration and the specific animal involved. Owen and Neal (1957) administered chlorpromazine intravenously (1mg/Kg), and intramuscularly (2mg/Kg), to a series of horses. They found after intravenous injection that 5 to 15 minutes elapsed before the full effects of the drug were noticeable, and residual effects lasted from 5 to 8 hours. For intramuscular injection the corresponding times were 45 to 60 minutes and 12 to 18 hours, and in some cases very slight effects were noted up to 48 hours. Martin and Beck (1956) reported that intramuscular doses of chlorpromazine greater than 2.5 mg/Kg produced greatest effects between 1 and 6 hours of injection and in some cases effects were still noticeable up to 48 hours.

After intravenous injection (~ 1 mg/Kg), promazine was found to produce noticeable effects within 5 minutes, the action

diminishing gradually over 2 hours (Raker and English, 1959). After a dose of 1.25 mg/Kg, Schulz (1958) reported the same time of onset, the effect lasting for 4 hours. Repeated daily doses did not appear to have any effect on the onset or duration of action. Gorman (1959) administered 50-500 mg. doses of promazine intramuscularly and noted an effect within five minutes which became most marked within the first hour. In some cases residual effects were noticed up to 24 hours. After parenteral administration of acepromazine predictable sedation has been produced within 15 minutes and residual effects have been noticed up to 8 hours (Van Laun, Nettleton and Japp, 1961).

From these facts the following observations can be made regarding the onset and duration of action of phenothiazine tranquillisers in the horse. After doses producing the same clinical effect their duration of action decreases in the order chlorpromazine > promazine > acepromazine. Effects of intravenous administration are noticeable within the first 5 to 15 minutes after injection, whereas, after intramuscular administration 45 minutes to 1 hour elapses before there is any noticeable response. Effects are most marked up to 8 hours depending on the dose and route of administration, and residual effects can be noticed up to 48 hours. Repeated daily doses do not seem to have any cumulative effect on duration of action.

CHEMICAL AND BIOCHEMICAL TRANSFORMATIONS

OF PHENOTHIAZINE AND ITS DERIVATIVES.

II. CHEMICAL AND BIOCHEMICAL TRANSFORMATIONS OF PHENOTHIAZINE AND ITS DERIVATIVES.

A. Chemistry of Phenothiazine.

Phenothiazine, when pure, is a light yellow crystalline solid (M.P. 180-181°C), soluble in most organic solvents, but almost insoluble in water, being wetted only with difficulty. It is a reactive compound giving rise to a vast range of derivatives, many of which have proved useful commercially, (Chapter 1). Its reactivity is most evident in its ease of oxidation, particularly in the presence of moisture. This accounts for the greyish or greenish blue colour of commercial preparations.

The compound and its derivatives act as weak bases, and have low ionisation potentials. The latter property is thought to be related to their physiological activity (Kearns & Calvin, 1961; Lyons and Mackie, 1963). They are strong electron donors and form weak complexes with molecules of high electron affinity by means of an intermolecular charge-transfer process. Many of these complexes are coloured, and their formation has been used as a method of colorimetric assay (Cupples, 1942; Payfer and Marshall, 1945; Stewart, 1947).

(1) Preparation of Derivatives - Nuclear Substitution.

Phenothiazine undergoes many direct nuclear substitution reactions, the most common being nitration, acylation, halogenation, metalation and mercuration. Nitration is usually accompanied by oxidation of the sulphur atom, the degree of nitration

depending on the conditions used. By heating phenothiazine with fuming nitric acid at $0^{o}C$, Bernthsen (1885) obtained two isomers, only one being identified (3,7-dinitro phenothiazine sulphoxide). Using milder conditions only the mono-derivative was obtained (3-nitro phenothiazine sulphoxide), and at higher temperatures trinitro sulphoxides (Kehrmann and Nossenko, 1913), and tetranitro sulphoxides have been obtained (Barnett and Smiles, 1910). Reduction of such compounds to their corresponding phenothiazine amines, followed by treatment with ferric chloride, gives rise to various members of the thiazine group of dyes (Bernthsen, 1885).

The nucleus is also readily acylated using the Friedel-Crafts reaction, but alkylation has not been reported by this method. The usual product is the 3,7-diacyl derivative (Massie, 1954), but N-acyl derivatives tend to orientate substitution to the 2- and 8- positions (Michels and Amstutz, 1950).

Direct halogenation has only been reported using chlorine, producing small amounts of 3,7-dichloro phenothiazine, a tetrachloro phenothiazine, and its sulphone (Ungar and Hoffman, 1896). Other halogens have been found to produce the corresponding phenothiazonium derivatives (Pummerer and Gassner, 1913). Ungar and Hoffman also reported a chlorination reaction using an ethereal solution of hydrogen chloride and nitrogen tetroxide, which produced 1,3-dichloro phenothiazine plus a mono- and a tetra-substituted derivative. This method also produced small yields using

hydrobromic acid, but no reaction was noted with hydriodic acid, (Page and Smiles, 1910). Halogenated derivatives are best prepared by ring closure methods using halogenated diphenylamines (Smith, 1950), or by replacement of a metal nuclear substituent (Gilman et al., 1954). The latter method, using either lithium or mercury, has been used as an intermediate step in the preparation of a variety of substituted phenothiazines, (Gilman et al., 1944, 1954; Finze, 1932).

(2) Ring Closure Methods.

In addition to direct substitution of the phenothiazine ring, nuclear substituted phenothiazines can also be prepared by ring closure methods, using starting materials already substituted with the required grouping. The most common technique follows Bernthsen's original synthesis of phenothiazine by fusing an appropriately substituted diphenylamine with sulphur. Para-substituted diphenylamines form 3-substituted phenothiazines, ortho-diphenylamines give the 1-substituted derivative, but meta compounds give rise to either 2- or 4- substituted phenothiazines, which are very difficult to differentiate chemically. Using this method alkyl phenothiazines have been prepared from the corresponding alkyl diphenylamines (Gilman and Shirley, 1944), and hydroxy phenothiazines from hydroxy diphenylamines (Houston, Kaster and De Eds, 1949). Alkoxy phenothiazines (Baltzly, Harfenist and Webb, 1946) and halogenated phenothiazines (Smith, 1950) have also been prepared in this manner. N-substituted

diphenylamines do not appear to undergo this thionation reaction (Massie, 1954).

Ring closure can also be effected using substituted diphenylamine-ortho-sulphinic acids. Krishna and Jain (1931) prepared 3-nitro phenothiazine by dissolving 4-nitro diphenylamine -2-sulphinic acid in sulphuric acid, and subsequently diluting this solution. Other substituted phenothiazines have been prepared by this reaction using acetic acid. (Warren and Smiles, 1932).

Nuclear substituted phenothiazines have also been prepared by condensation of o-amino or o-amido thiophenols with various nitrobenzenes. Kehrmann and Steinberg (1911) condensed o-amino thiophenol hydrochloride with picryl chloride to give 1,3-dinitro phenothiazine. Many substituted derivatives have been prepared using the same reaction (Baltzly et al., 1946). Such reactions have been shown to proceed through a rearrangement common to 2-nitro-2'-aminodiphenyl sulphides, which in the presence of alkali rearrange to 2-nitro-2' mercapto diphenylamines (Evans and Smiles, 1935a, 1935b; Wight and Smiles, 1935).

(3) N-substitution.

The interest in nuclear substituted phenothiazines of industrial importance was largely confined to the first half of this century. Recently interest has tended more to the synthesis of N-substituted

derivatives, especially N-alkylaminoalkyl phenothiazines, due to their widespread therapeutic properties (Chapter 1).

The nitrogen atom can be alkylated using either alcohols, sulphates or halides. However, only the 10-methyl and the 10-ethyl derivatives can be prepared using alcohols. The reaction involves heating phenothiazine, dry hydrogen chloride, and the appropriate alcohol in a sealed tube (Bernstein and Rothstein, 1944). Likewise only methyl sulphate has been found useful as an N-alkylating agent (Gilman and Nelson, 1953).

More important are the condensation reactions, involving alkyl or aryl halides, which normally require a basic medium. In initial studies Gilman and Shirley (1944) prepared a series of long chained 10-alkyl phenothiazines, and a series of 10-aryl phenothiazines, by condensing phenothiazine and the corresponding halide in the presence of sodium carbonate, with copper powder as catalyst. However, further attempts at condensation using alkylaminoalkyl halides were unsuccessful.

10-alkylaminoalkyl phenothiazines were subsequently prepared by Charpentier (1947, 1952) by condensing phenothiazine and the corresponding halide in the presence of sodamide. Using the same condensing medium Dahlbom (1949) prepared piperidyl alkyl phenothiazines by reaction with the appropriate halide. This method has also been used for the preparation of morpholinyl alkyl phenothiazines and N-pyrrolidyl alkyl phenothiazines (Reid, Wright, Kolloff and Hunter, 1948). Other methods used for the preparation

of aminoalkyl phenothiazines have included reaction with Grignard complexes (Berg and Ashley, 1953), or reaction of alkylamino halides with 10-lithio phenothiazine (Gilman and Shirley, 1944).

(4) Oxidation of Phenothiazine.

The most important property of phenothiazine, from a biological point of view, is its ease of oxidation. The products are shown in Figure 1. It readily forms the sulphoxide (B) and the sulphone (C) on reaction with such oxidising agents as potassium permanganate or hydrogen peroxide (Massie, 1954). Sulphoxide formation has also been reported on irradiation of an alcoholic solution of phenothiazine (Brown, 1955). The compound can also be oxidised to various hydroxylated and quinonoid structures (Kehrmann, 1906; De Eds and Eddy, 1938a). The nucleus can be hydroxylated at one position para to the nitrogen atom to give the colourless compound leucophenothiazone (D), which, in aqueous solution, is readily oxidised by the atmosphere to the intensely coloured compound, phenothiazone (E). Hydroxylation at both positions para to the nitrogen atom produces another colourless compound leucothionol (F), which is also readily oxidised by air and moisture to a deep red compound, thionol (G).

These two systems, leucophenothiazone-phenothiazone and leucothionol-thionol have been shown to be based on redox reactions, proceeding, in acid solution, through a stable free radical intermediate (De Eds and Eddy, 1938b; Granick, Michaelis

Fig.1 PHENOTHIAZINE AND ITS OXIDATION PRODUCTS

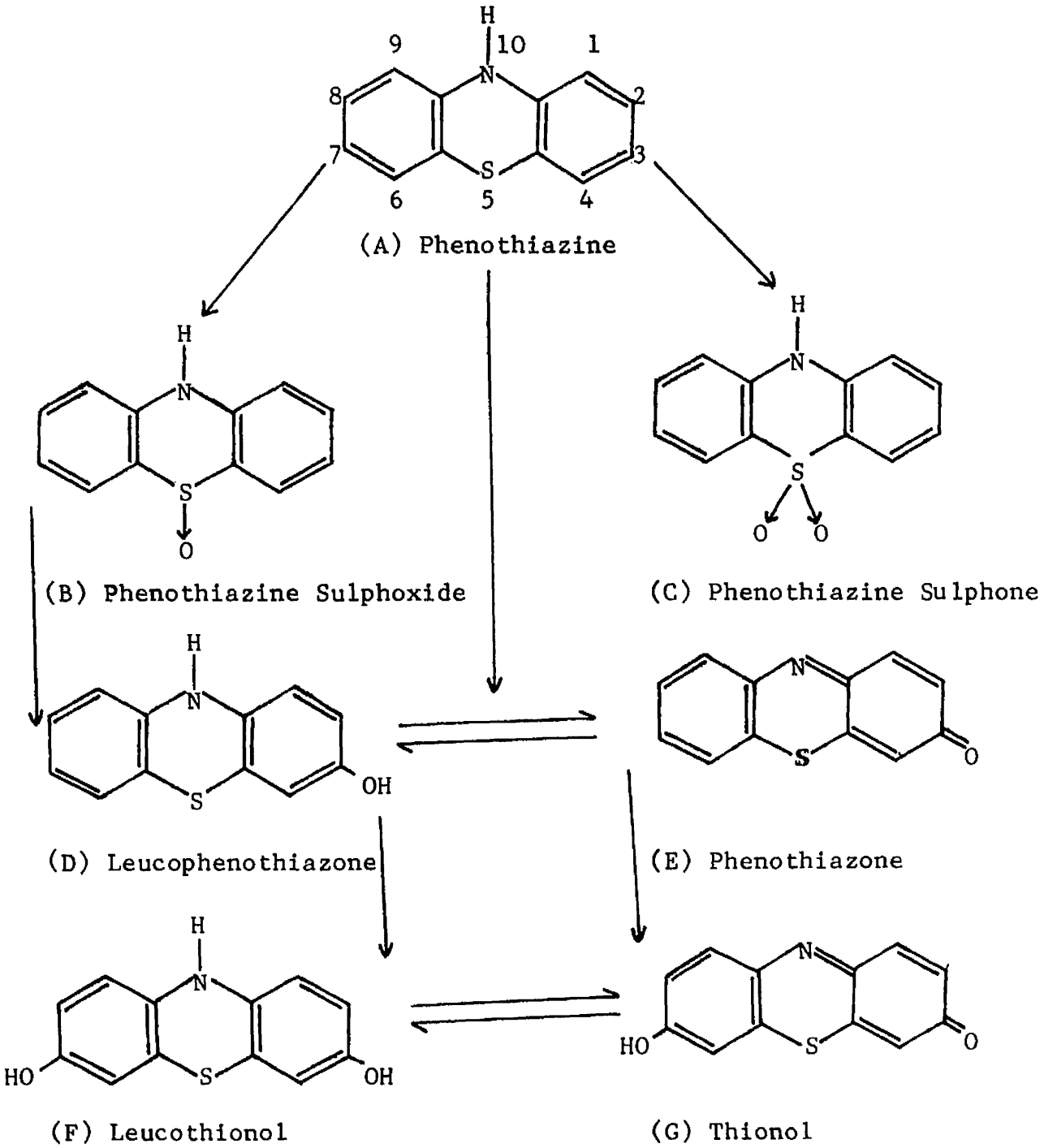
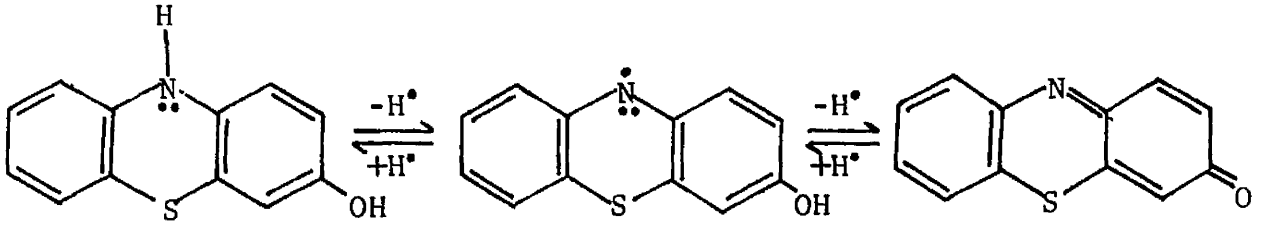
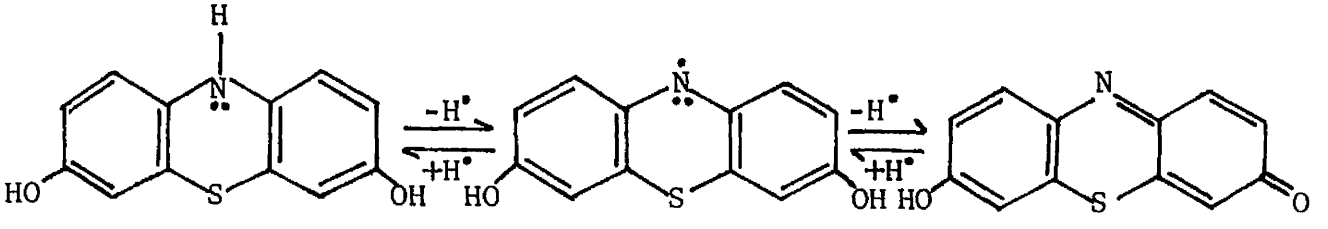


Fig.2a FREE RADICAL INTERMEDIATES IN THE SYSTEMS LEUCOPHENOTHIAZONE-PHENOTHIAZONE AND LEUCOTHIONOL-THIONOL (GRANICK et al.,1940)

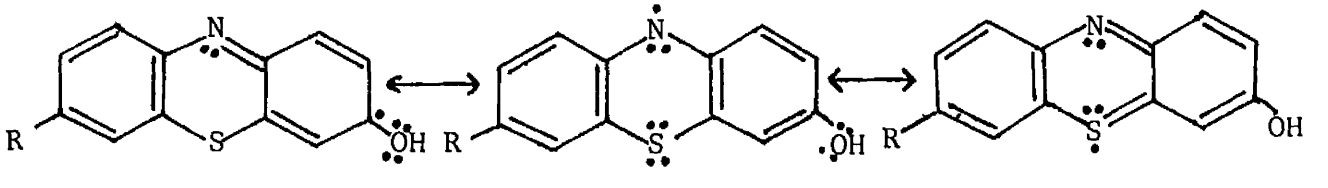


Leucophenothiazone Phenothiazone



Leucothionol Thionol

Fig.2b STABLISATION OF THE FREE RADICAL BY RESONANCE IN ACID SOLUTION



R = H or OH

and Schubert, 1940; Granick and Michaelis, 1947). In strongly acid solutions the intermediate is stabilised by resonance, but as the pH increases the stability of the free radical decreases, until the oxidation seems to take place in a single step. (Figure 2).

A relationship between the various oxidation products has been described, and explained by a postulated rearrangement of the sulphoxide to the hydroxylated form, (Craig and Tate, 1961). Details of the rearrangement are given in Figure 3. The first stable oxidation product of phenothiazine is phenothiazine sulphoxide (3), (Gilman and Shirley, 1944). This product is in tautomeric equilibrium via B1 and B2 with compound B3, which has been shown to be a phenazothionium compound by its isolation as the phenazothionium salt of various anions (Pummerer, Eckert and Gassner, 1914; Barnett and Smiles, 1909; Kehrmann and Christopoulos 1921). The cation of B3 then undergoes acid-catalysed rearrangement via the transition stage B4 to the mesomeric form B5, which causes a shift of the anionic group to the 3-position to produce the pseudo-base, (B6). The molecule is finally stabilised by migration of a proton from the 3-position to give leucophenothiazone, (D), (Kehrmann, 1902). Leucophenothiazone (D) is readily oxidised by air to the red dye phenothiazone, (E), and it has been postulated that (E) can be further oxidised via the sulphoxide to leucothionol, (F), and finally thionol, (G), (Craig and Tate, 1961).

This rearrangement of phenothiazine sulphoxide to leucophero-

FIGURE 3

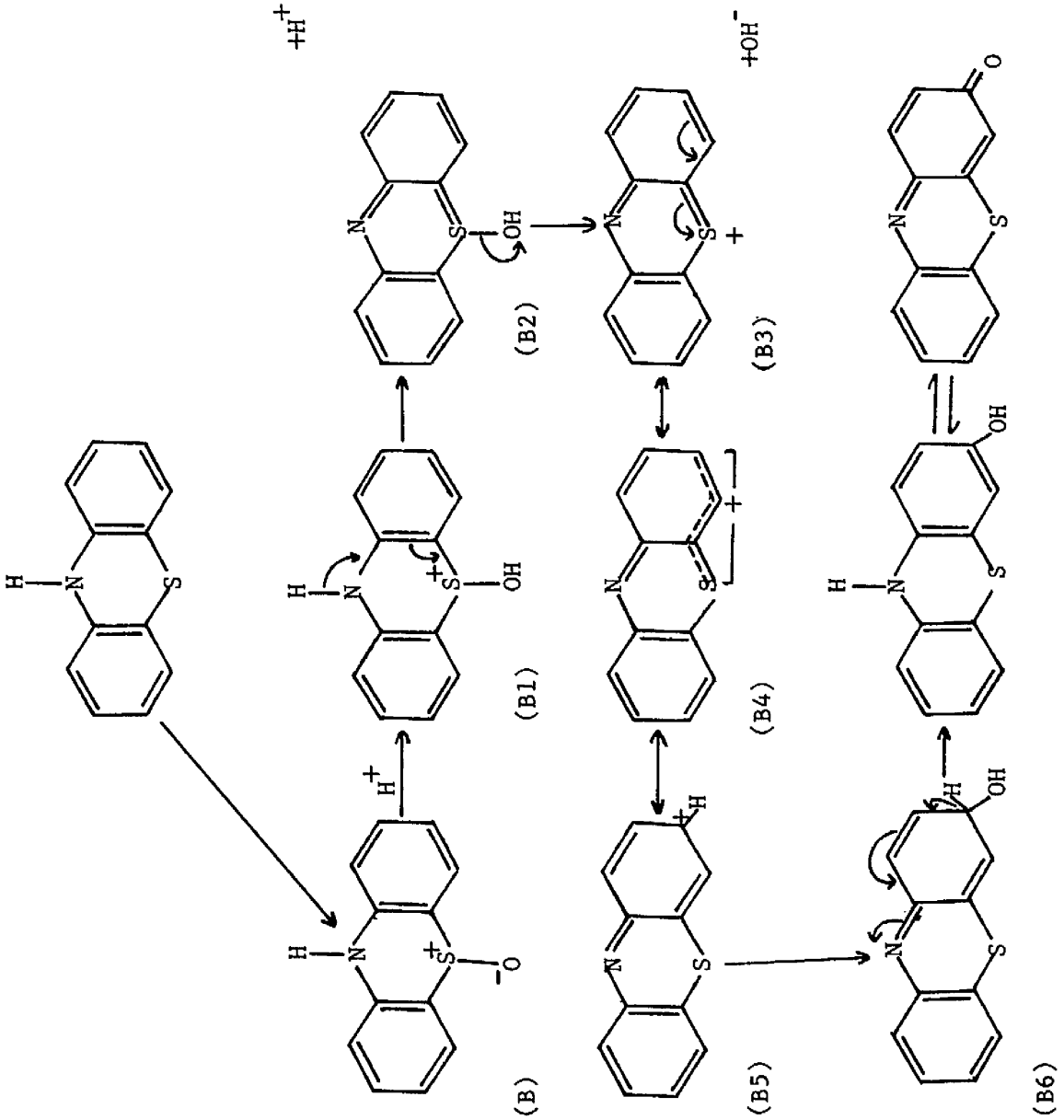


Fig.3. REARRANGEMENT OF PHENOTHIAZINE SULPHOXIDE TO LEUCOPHENOTHIAZONE (CRAIG & TATE, 1961).

thiazone has been shown to take place in the presence of acetic acid (Hilditch and Smiles, 1912). However, when hydrochloric acid was used the product was 3-chlorophenothiazine, and in the case of 10-alkylphenothiazine sulphoxides the rearrangement has been shown to produce 3-chloro-10-alkyl-phenothiazines (Page and Smiles, 1910; Gilman et al., 1954; Schmalz and Burger, 1954; Kehrmann and Nossenko, 1913). The rearrangement does not take place when the 3- and 7- positions are blocked, (Hilditch and Smiles, 1912; Page and Smiles, 1910).

B. Metabolism of Phenothiazine.

Initial metabolic studies on phenothiazine provided the groundwork for our present day knowledge of the metabolism of its derivatives. The biotransformations which it undergoes in the body are basically the oxidation reactions encountered by the chemist in the laboratory.

Its metabolism has been extensively studied in a variety of species. These include sheep (Lipson, 1940; Swales and Collier, 1940; Collier, Allan and Swales, 1943; Clare, 1947; Harpur, Swales and Denstedt, 1950), cattle (Clare, Whitten and Filmer, 1947; Whitten, Clare and Filmer, 1946; Whitten, 1947, Whitten and Filmer, 1947; Duthby and Lewis, 1949; Ellison and Todd, 1957; Richardson and Todd, 1958), swine (Clare, 1947; Collier et al., 1943), horses (Collier et al., 1943), dogs (Collier et al., 1943; De Eds and Thomas, 1941), rats (De Eds, Eddy and Thomas, 1938; De Eds and Thomas, 1942), rabbits (De Eds, Eddy and Thomas, 1938; De Eds and Thomas, 1941; De Eds and

Thomas, 1942; Benham, 1945; Collier et al., 1943) and man (De Eds and Thomas, 1941, 1942; De Eds et al., 1938). Initial studies were carried out on urine since it was noted that the urine of animals dosed with phenothiazine took on a reddish coloration, suggesting transformation of the administered drug. Also, although phenothiazine is practically insoluble in water, sheep were found to eliminate 40% of a dose in the urine within 48 hours (Harpur, Swales and Denstedt, 1950).

De Eds and Thomas (1942) showed the presence of leucophenothiazone and phenothiazone, along with small amounts of leucothionol and thionol, as metabolic products of phenothiazine in the urine of rabbits, rats and man. Such oxidation products would account for the coloration observed in the urine of dosed animals. Leucophenothiazone and leucothionol were also detected as urinary metabolites in sheep (Lipson, 1940). De Eds et al. (1938) also found phenothiazine as an acid-labile conjugate in the urine of rats and man. This was later reported to be the major metabolic product of phenothiazine in swine and man (Craig and Tate, 1961). The latter workers also suggested that, since phenothiazine is a weak base, and therefore would not form salts under biological conditions, the conjugation probably takes place at the nitrogen atom through an acid-labile amide bond.

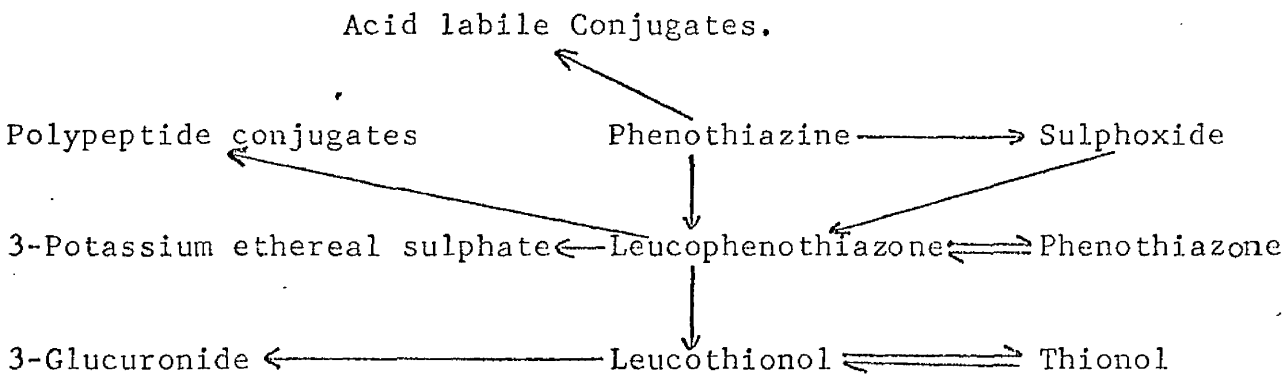
Further studies showed that phenothiazine and its oxidised derivatives (Figure 4) are excreted as metabolites in the urine, either in the free or a conjugated form. However, the relative

amounts of each varies from species to species. Collier and co-workers carried out a series of urinary excretion studies on sheep, dogs, horses and rabbits, (Collier, 1940; Collier et al. 1943). Leucophenothiazone which they identified by synthesis, was found to be the major metabolite in sheep, dogs and horses, mostly as the ethereal sulphate. Traces of leucothionol were also detected. However in rabbits, leucothionol was found to be the predominating metabolite. This was confirmed by Benham (1945) who also reported that the leucothionol is excreted as a conjugate with glucuronic acid. Leucophenothiazone has since been detected as the ethereal sulphate in the urine of dosed cattle (Ellison, Todd and Harvey, 1957).

Although in some cases, it is only the leuco bases of phenothiazone and thionol that are excreted in the urine, these are oxidised on exposure to the air to the coloured quinonoid compounds. Due to the reversible redox systems operating with these compounds, it is usually found that this coloration gradually disappears due to the action of reducing compounds in the urine (Clare, 1947).

A further metabolite of phenothiazine, phenothiazine sulphoxide, was discovered by Clare et al. (1947) in the blood of sheep and calves. They postulated that the sulphoxide is formed in the alimentary tract and absorbed into the portal blood. It is then transformed by the liver to leucophenothiazone, conjugated as the ethereal sulphate, and excreted in the urine in this form.

Fig. 4. MAJOR METABOLIC PRODUCTS OF PHENOTHIAZINE.



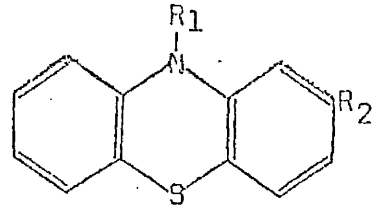
Leucophenothiazone and leucothionol were also found in the urine along with an unidentified conjugate of phenothiazine.

With high doses of phenothiazine, the sulphoxide is not completely transformed by the liver, and reaches the systemic circulation and aqueous humour of the eye. This also occurs with calves after low doses, causing a photosensitised keratitis (Clare, 1947). In calves less than six weeks old, Clare also found leucophenothiazone in the urine in association with an unidentified polypeptide. Sulphoxide formation was later shown to be a major metabolic pathway of the pharmacologically active 10-substituted phenothiazines. A flow diagram of the main metabolic pathways of phenothiazine is shown in Figure 4. The subject has been extensively reviewed by Harwood (1953), Craig and Tate (1961), Findlay (1950).

C. Chemistry of the Tranquillisers.

As previously described (Pg. 18) the tranquillisers form a homologous series with the general formula shown, the numbering convention being the same as for phenothiazine. Classification is based on the substituents R_1 (an aminoalkyl side chain) and R_2 (an electronegative atom or group). They are viscous oily liquids with an amine odour and an alkaline reaction, but are available commercially in crystalline form as their salts. Promazine is manufactured as the hydrochloride taking the form of white hygroscopic crystals decomposing at 191°C. Chlorpromazine, also available as the hydrochloride, decomposes at 194-196°C. Acepromazine, which is marketed as the maleate, has a melting

point of 135-136°C. Unlike the parent compounds, these salts are readily soluble in water giving a slightly acid solution. They are also soluble in methanol, ethanol and chloroform, but only slightly soluble in ether and benzene.



Chemically, they are very reactive, with properties similar to phenothiazine, which form the basis of a large number of colour reactions widely used in their detection and determination (Pg. 68). Their reactivity is also borne out by the large number of metabolic changes which they undergo (Pgs. 51 - 66).

(1) Oxidation and Stability.

Like phenothiazine, their nucleus is susceptible to oxidation, and sulphoxides and sulphones are readily formed by reaction with peroxide or permanganate (Massie, 1954). However, substituents at the 2- and 10- positions exert a marked influence on the oxidative processes, affecting the course and kinetics of such reactions. This influence is also a controlling factor on their stability in solution.

Kabasakalian and McGlotten (1959) determined the anodic oxidation half-wave potentials of a series of such compounds, and reported that their stability increases with an increase in

electronegativity of the 2-substituent. They also stated that the stability is influenced by the group on the 10-position, although the precise effect is not predictable. Similar findings were reported by Bolt (1965).

The stability of several phenothiazine tranquillisers in an aqueous solution was investigated by Waaler (1960). On refluxing solutions of promethazine (R_1=-$CH_2CH(Me)NMe_2$), or ethopropazine (R_1=-$CH_2CH(Me)NEt_2$), decomposition took place to 10-methyl phenothiazine, acetaldehyde, and the corresponding dialkylamine. Solutions of promazine or chlorpromazine (R_1=-$(CH_2)_3NMe_2$), on the other hand, showed considerable stability.

Persulphate oxidation of phenothiazine derivatives was studied by Nano and co-workers. In initial experiments they showed that promethazine is oxidised to phenothiazone along with other unidentified products (Nano and Sancin, 1961). They believed that the reaction proceeded by cleavage of the side chain to form phenothiazine, followed by oxidation of the nucleus to phenothiazone.

At first they thought this cleavage peculiar to promethazine, and that other derivatives were oxidised by a different unspecified mechanism. However, in a subsequent investigation, (Nano, Sancin and Tappi, 1963), they studied the reaction on a series of alkylaminoethyl phenothiazines, and showed that derivatives possessing a two carbon polymethylene chain between the nuclear and side chain nitrogen atoms were oxidised to phenothiazone and other unidentified

quinonoid structures. Those with a three carbon polymethylene chain were oxidised to the corresponding sulphoxide, only trace amounts of quinones being found. Pungor (1960) investigated the persulphate oxidation of chlorpromazine and reported the major reaction product to be a dimer of molecular weight 500 in which both aminoalkyl side chains are intact.

(2) Irradiation and free radical formation.

The nucleus is also liable to photochemical oxidation, which is apparent from the intense colours experienced when solutions of such drugs are exposed to light, (Holmes and Snyder, 1929; Reid, 1930; Necrae, 1931; Brown, Cole and Crowell, 1955; Ravin, Kennon and Swintosky, 1958). The latter workers related the photochemical oxidation of chlorpromazine to an uptake of oxygen during irradiation. Brown et al. (1955) irradiated an alcoholic solution of phenothiazine, and isolated and identified both phenothiazone and phenothiazine sulphoxide. Beckett, Beaven and Robinson (1963) stated that the major product from irradiation of aqueous solutions of chlorpromazine is a dimer in which both chlorine atoms have been lost. At least twelve products were reported by Huang and Sands (1964) after irradiation of aqueous chlorpromazine solutions, of which two were identified as chlorpromazine sulphoxide and chlorpromazine N-oxide (Fig. 6). They also noted that several other products gave blue or purple colours with 50% sulphuric acid, which Bolt

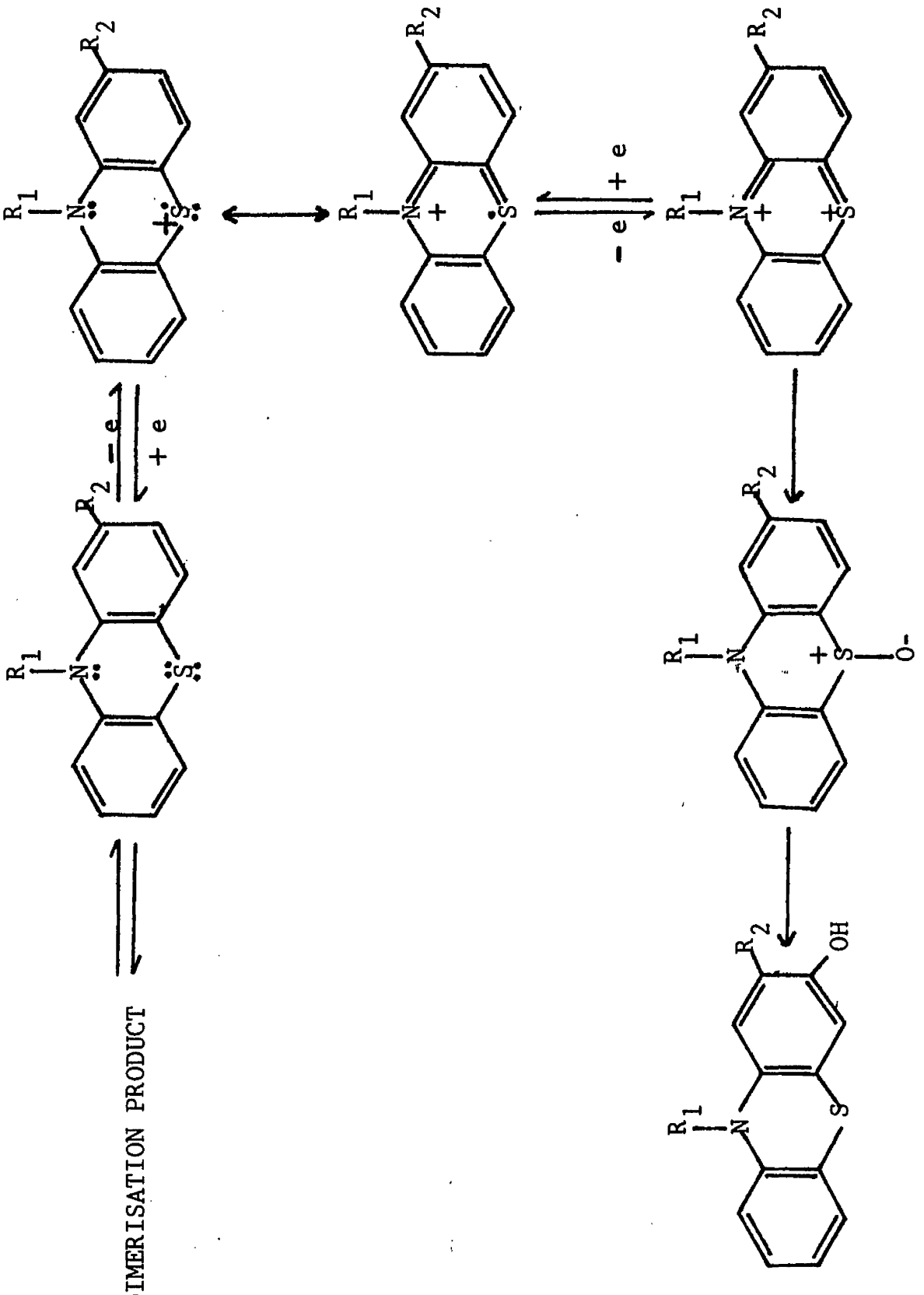
(1965) attributed to combinations of hydroxylation and dechlorination of the phenothiazine nucleus.

Many reports of the formation of free radicals during the oxidation of phenothiazine derivatives have appeared in the literature, and it is now believed that such species play an important role in the oxidation process. Forrest, Forrest and Berger (1958), using electron spin resonance spectroscopy, showed the presence of free radicals after ultra violet irradiation of aqueous solutions of chlorpromazine, and claimed that the intermediate formed was identical to one of the urinary metabolites of the drug (Pg. 64). Such species were also reported by Fels and Kaufmann, (1959) during the oxidation of acidic chlorpromazine solutions by ferric salts. Piette and Forrest (1962) subsequently claimed that the first oxidation products of phenothiazine drugs are free radicals.

Borg and Cotzias (1962) studied the oxidation of chlorpromazine solutions during titration by metal ions, and, by following the reaction potentiometrically, demonstrated that the process proceeds through a free radical intermediate. The first step is loss of an electron to form a free radical ion, which in turn loses an electron to give a dipositive phenothiazonium ion. Reaction of the latter with water gives rise to chlorpromazine sulphoxide. The reaction, however, is further complicated by interaction of the radical and unchanged chlorpromazine leading to dimerisation of the chlorpromazine and disproportionation of the free radical to both chlorpromazine and chlorpromazine

FIGURE 5

Fig. 5 FREE RADICAL INTERMEDIATES IN THE OXIDATION OF SUBSTITUTED PHENOTHIAZINES



sulphoxide (Figure 5).

A comparable mechanism for photochemical oxidation was subsequently formulated by Felmeister and Discher (1964), who also claimed that irradiation of aqueous chlorpromazine solutions results in formation of free radicals, which disproportionate to chlorpromazine and chlorpromazine sulphoxide. They also reported that one of the reaction products could be acylated, and concluded that hydroxylation had occurred at the 3-position, possibly by rearrangement of the sulphoxide (c.f. Pg. 38). Figure 5 shows a general mechanism of oxidation of substituted phenothiazines based on this information.

These investigations bear out the complexity of the oxidation reactions of the phenothiazine tranquillisers and it is evident that information on this subject is still incomplete. Although sulphoxide and sulphone formation are known to occur, other oxidation reactions are not so well defined as for phenothiazine. The compounds produce intense colours on oxidation which provide a useful method for their detection but the course and ease of such reactions are difficult to predict or determine. The formation of free radical intermediates, and resulting polymers has been reported, but it is still not certain whether substituted products equivalent to thionol or phenothiazone are present. Berti and Cima (1956) stated that the formation of such compounds is unlikely as the substituted nitrogen would prevent the shift in double bond necessary for the reaction.

(3) Side Chain Transformations.

The side chain at the 10-position is also susceptible to several modifications. Oxidation of the terminal nitrogen atom to the N-oxide has been claimed by Japanese workers, although the reaction was not proved conclusively (Sumita and Nishino, 1960, a,b,c). Chlorpromazine N-oxide has been identified after irradiation of aqueous chlorpromazine solutions (Huang and Sands, 1964). N-oxide formation has also been shown to be a metabolic pathway of both chlorpromazine and imipramine, a compound chemically related to the tranquillisers (Fishman, Heaton and Goldenberg, 1962; Fishman and Goldenberg, 1962). The side chain has also been shown to be subject to demethylation and cleavage as a result of biochemical oxidation (Pg. 62).

D. Metabolism of the Tranquillisers.

Due to their widespread use both in psychiatry and general medicine, the metabolism and excretion of these drugs has been the subject of much research over the past 15 years. Results so far have underlined the complexity of such processes, and have shown marked variations from species to species (Goldenberg and Fishman, 1961; Goldenberg, Fishman, Heaton and Burnett, 1964).

Five major pathways of metabolism have been found, namely, oxidation of the sulphur atom to the sulphoxide (Salzman and Brodie, 1956; Fishman and Goldenberg, 1960), oxidation of the terminal side chain nitrogen atom (Fishman, Heaton and Goldenberg,

1962), demethylation of the side chain amino group (Ross, Young and Maass, 1958; Walkenstein and Seifter, 1959), and hydroxylation of the phenothiazine nucleus, usually followed by conjugation with glucuronic or sulphuric acid (Lin, Reynolds, Rondish and Van Loon, 1959; Huang, Sands and Kurland, 1961; Beckett, Beaven and Robinson, 1963; Fishman and Goldenberg, 1963). These transformations can take place singly, or in combination, giving rise to a large number of possible metabolites. At least thirty urinary metabolites of promazine have been detected after oral administration to humans (Goldenberg et al., 1964). Studies have shown extensive localisation of such compounds in the body (Salzman and Brodie, 1956; Fyodorov, 1958; Walkenstein and Seifter, 1959), low percentage excretion of dose (Fyodorov and Shnol, 1956; Nadeau and Sobolewski, 1959; Beckett et al., 1963; Goldenberg et al., 1964), and prolonged excretion sometimes lasting several months after administration (Forrest, Forrest and Mason, 1959; Huang et al., 1961).

(1) Sulphoxide formation.

This was the first important metabolic route to be discovered. Salzman and Brodie (1956), using counter current distribution and paper chromatography, isolated and identified chlorpromazine sulphoxide as a major urinary metabolite of chlorpromazine in dog and man. They detected at least three metabolites, only the sulphoxide being identified. They also claimed that the drug

was extensively localised in various tissues, especially the brain, accounting for the low plasma concentration ($\sim$ 2µg/ml) obtained 30 minutes after injection. In the dog 10-14% of an intravenous dose (20 mg/Kg) was excreted as the sulphoxide, and less than 2% as unchanged drug. After oral administration to man, only 5% was excreted as the sulphoxide along with trace amounts of the parent drug. They postulated that the sulphoxide had been further metabolised to unidentified products, since, after administration of chlorpromazine sulphoxide to dogs, only 20% of the dose was recovered in the urine unchanged (Salzman, Moran and Brodie, 1955). Similar findings have been reported in man where only 2-3% of an intravenous dose of this metabolite was recovered unchanged (Davidson, Terry and Sjoerdsma, 1957).

Sulphoxidation has since been extensively reported. Huang and Kurland (1961) showed that, after repeated administration of chlorpromazine to humans, urinary excretion of sulphoxide metabolites varied between 1 and 18% of the daily dose, less than 1% being recovered as unchanged drug. Goldenberg and Fishman (1961) reported that after oral administration of chlorpromazine to man and dog 5.9 and 11% of the dose respectively were excreted in the urine as sulphoxides. Again the unchanged drug was detected in only trace amounts. Sulphoxide formation has also been shown to occur in mice (Christensen and Wase, 1956), rats (Ogawa, Kawasaki and Yamamoto, 1958; Emmerson and Miya, 1962) and rabbits (Berti and Cima, 1956; Goldenberg et al., 1964).

Although initial investigations and most subsequent studies of this metabolic pathway were primarily concerned with chlorpromazine, sulphoxidation is also a common metabolic pathway of promazine (Walkenstein and Seifter, 1959), mepazine (Hoffman, Nieshulz, Popendiker and Tauchert, 1959), methoxypromazine (Allgén, Jönsson, Rappe and Dahlbom, 1959), promethazine (Hansson and Schmiterlow, 1961), perphenazine (Symchowicz, Peckham, Korduba and Pertman, 1962), levomepromazine (Allgén, Hellstrom and Sant'orp, 1963), and such closely related compounds as thioridazine, (Zehnder, Kalberer, Kreis and Rutschmann, 1962) and chlorprothixene (Allgén, Jönsson, Nauckhoff, Anderson, Huus and Nielsen, 1960).

Sulphoxidation is now generally accepted as one of the major metabolic pathways of the phenothiazine tranquillisers although the sulphoxide itself accounts for only a small percentage of the dose. Sulphone formation, on the other hand, does not appear to take place in vivo, although it has been reported for the related compounds methylene blue in cats, dogs and rabbits. (Underhill and Closson, 1905), and thioridazine in rats. (Zehnder et al., 1962). It has been postulated that chlorpromazine sulphone may be formed in vitro using fortified liver homogenates (Kamm, Gillette and Brodie, 1958).

(2) Demethylation.

Demethylation of the terminal side chain amino group was first reported by Ross et al.. (1958). They administered chlorpromazine,

(labelled at the N-methyl group with ^{14}C), to rats, collected the expired CO_2 and measured its radioactivity. 12-16% of the administered radioactivity was detected within 6 hours, and $^{14}CO_2$ was expired at the rate of 0.7-2% per hour over the 12 hours of the experiment. In the following year they demonstrated the demethylation of labelled chlorpromazine *in vitro* using fortified rat and rabbit liver homogenates (Young, Ross and Maass, 1959).

Walkenstein and Seifter (1959) investigated the urinary metabolites and excretion of ^{35}S promazine by both dogs and rats, and reported that demethylation could take place in combination with sulphoxidation. After intraperitoneal administration ($\sim$20mg/Kg) to dogs, they identified three urinary metabolites, promazine sulphoxide (3-5%), desmethylpromazine (0.75-1%) and desmethylpromazine sulphoxide (2.5 - 3.5%) in addition to the unchanged drug (2-3%). Also, on administration of either desmethyl-promazine or promazine sulphoxide to the animals, desmethyl-promazine sulphoxide was always found in the urine. They suggested that promazine is separately demethylated and oxidised to desmethylpromazine and promazine sulphoxide, which are then oxidised or demethylated respectively to form the third metabolite, desmethylpromazine sulphoxide. They also noted in dogs, that within 15 minutes of intraperitoneal injection half the radioactivity detected in the blood was due to promazine metabolites, although 7 or 8 days elapsed before 70-80% of the radioactivity was detected in the urine. The remainder was excreted in the faeces.

Radioactivity was also detected in the blood up to 4 days after injection. High degrees of localisation were also found in various organs (lung, liver, kidney, spleen) from both species (c.f. Salzman and Brodie, 1956).

The metabolism of ^{35}S chlorpromazine by rats was investigated by Emmerson and Miya (1962). They found 50% of the administered radioactivity in the urine over three days, the remainder being excreted in the faeces. Three metabolites were identified in the urine, chlorpromazine sulphoxide (5%), and the sulphoxides of desmethylchlorpromazine (5%), and desdimethylchlorpromazine (2%). Chlorpromazine recovered accounted for approximately 12% of the dose.

Further evidence for the combination of sulphoxidation and demethylation was furnished by Fishman and Goldenberg (1960). In the first of a series of studies into tranquilliser metabolism they detected the presence of 10 metabolites of chlorpromazine in human urine. Using paper chromatography, specific spray reagents, and ultra violet spectrometry, they showed that six were sulphoxides, two being demethylated, (desmethylchlorpromazine sulphoxide and desdimethylchlorpromazine sulphoxide).

The following year they carried out further investigations of these metabolites in dog and man (Goldenberg and Fishman, 1961), and showed both qualitative and quantitative excretion differences between the species. In man desdimethylchlorpromazine sulphoxide was the major metabolite (3.7%), followed by desmethylchlorpromazine sulphoxide (1.8%), and chlorpromazine sulphoxide (0.4%), whereas

in the dog desmethylchlorpromazine sulphoxide (5.1%) and chlorpromazine sulphoxide (5.1%) predominated. Only 1.1% was excreted as the didesmethylated derivative. Unchanged drug accounted for 0.2 and 2.8% respectively. Desmethylchlorpromazine was detected in dog urine, but was found to be absent in humans.

(3) N-oxide formation.

A further metabolite of chlorpromazine, chlorpromazine N-oxide was identified by Fishman et al. (1962) in the urine of dog and man. Although it is not a major metabolite in humans it was found in greater quantity (0.7%) than the sulphoxide (0.4%) and was even more abundant in the dog (2-3.5%). N-oxide formation has also been shown to be a metabolic pathway of promazine (Goldenberg et al., 1964), and imipramine (Fishman and Goldenberg, 1962).

(4) Hydroxylation and Conjugation.

The derivatives already described are all readily extractable from urine at an alkaline pH and are referred to as the non-polar metabolites. However, a further group exists, present in much greater abundance in most species, which are not so readily extracted by common organic solvents. Known collectively as the polar metabolites, the group consists of many hydroxylated and conjugated derivatives. Goldenberg and Fishman (1961) reported that such derivatives are the predominating metabolites in man, and that qualitative and quantitative differences also exist in this fraction from species to species.

In 1959, Flanagan and co-workers, using chromatographic techniques, reported that chlorpromazine, administered intraduodenally to dogs, was excreted in the bile and urine as "free" and "bound" chlorpromazine sulphoxide, (Flanagan, Lin, Novick, Rondish, Bocher and Van Loon, 1959). This was demonstrated by the fact that strong alkaline hydrolysis, or β -glucuronidase incubation, gave rise to an additional group of ether extractable metabolites. The "bound" fraction was found to predominate in both urine and bile, the ratios being approximately 3:1 and 12:1 respectively. The nature of the binding substances were not identified.

In the same year Nadeau and Sobolewski reported similar results for humans after administration of chlorpromazine or levomepromazine. Since both chemical hydrolysis and β -glucuronidase incubation produced similar results they assumed that the main binding constituent was glucuronic acid. 10-20% of the chlorpromazine and 5% of the levomepromazine was recovered over 24 hours, and 50-98% of the metabolites were glucuronide conjugates.

Further evidence for the presence of conjugated was furnished by Lin and co-workers who isolated chlorpromazine metabolites using a cation exchange resin, then liberated glucuronic acid by incubation with β-glucuronidase (Lin, Reynolds, Rondish and Van Loon, 1959). The metabolites released were shown to possess hydroxyl functions, and it was suggested that hydroxylation of chlorpromazine followed by conjugation with glucuronic acid is an important metabolic route of the drug in humans.

Many reports of hydroxylation and subsequent glucuronide formation have since appeared. Posner and co-workers found three hydroxylated derivatives of both promazine and chlorpromazine in human urine, and suggested that both monohydroxylation (at the 3-position) and dihydroxylation (at the 3,7 positions), followed by conjugation, had taken place (Posner 1959; Posner, Culpan and Levine, 1963, a,b). Huang and co-workers reported that phenolic derivatives and their glucuronides were the major metabolic products of chlorpromazine in man (Huang and Kurland, 1961, a,b; Huang, Sands and Kurland, 1961). Several glucuronides of chlorpromazine were isolated from the urine of psychiatric patients by Beckett et al. (1963). They claimed that the N-demethylated glucuronides were the major metabolic products of chlorpromazine in man, but the position of the glucuronide group was not firmly established. They detected 7% of the dose in 48 hours, glucuronides predominating over non-polar metabolites in the ratio of 2:1.

A series of phenolic derivatives of chlorpromazine has been identified in the urine of man and dog (Fishman and Goldenberg, 1963; Goldenberg et al., 1964). Using paper, thin layer and gas liquid chromatography, and comparison with known standards, they demonstrated that each member of the series was hydroxylated at position 7, and could be found both in the free form and as conjugates. Half of the series were sulphoxides, and in certain cases demethylation had occurred.

They also carried out a detailed study of the metabolism

of promazine in dogs, rabbits and man (Goldenberg et al., 1964). Hydroxylation was found to take place at the 3-position, and both free and conjugated forms were present, associated with various combinations of sulphoxidation and demethylation. In man 33% of the dose was recovered as 30 derivatives, the ratio of phenolic to non-phenolic metabolites being 1.4:1. In the phenolic fraction glucuronide conjugates predominated accounting for 15% of the dose, the unconjugated phenols representing only 1-2%. The unchanged drug accounted for only 0.2% of the dose and the major metabolites were desdimethylpromazine sulphoxide (6.3%), desmethylpromazine sulphoxide (5.2%), and 3-hydroxypromazine glucuronide (3.7%). Much smaller amounts of metabolites were recovered from both dogs (13%) and rabbits (10-13%). Qualitative differences were also observed amongst the three species.

In a subsequent study (Goldenberg and Fishman, 1965) they confirmed that positions 3- and 7- were the main sites of hydroxylation of promazine and chlorpromazine respectively. However, they also noted that hydroxylation of chlorpromazine at the 3-position is possible, and that dihydroxylation of both compounds at the 3-, 7- positions can occur.

Hydroxylation followed by glucuronide formation is now regarded as the major metabolic pathway for the tranquillisers in most species, and besides promazine and chlorpromazine has been reported for several other derivatives, (Padeau and Sobolewski, 1959; Herrmann, Schindler and Pulver, 1959; Herrmann and Pulver, 1960; Zehnder et al., 1962).

Other types of conjugates, formed to a lesser extent, have been reported by various workers. Beckett et al.. (1963) showed the presence of sulphate conjugates of chlorpromazine in human urine by aryl sulphatase incubation. 7% of the administered dose was recovered over 48 hours, consisting of non-polar metabolites ($\sim$ 25%), glucuronide conjugates ($\sim$ 60%) and sulphate conjugates ($\sim$ 19%). The free metabolites were excreted mostly as sulphoxides, whereas the conjugates were in the sulphide form. Other unidentified conjugates were liberated in small amounts by acid hydrolysis.

Ethereal sulphates of promazine were also detected in the urine of dogs, rabbits and man, after oral administration, by incubation with Mylase P (Goldenberg et al., 1964). In man the sulphate fraction accounted for $\sim$ 3% of the dose, the ratio of glucuronides, sulphates and unconjugated metabolites (including free phenols) being 5:5:1. Small amounts of acetylated phenolic derivatives were also found.

(5) Additional Transformations.

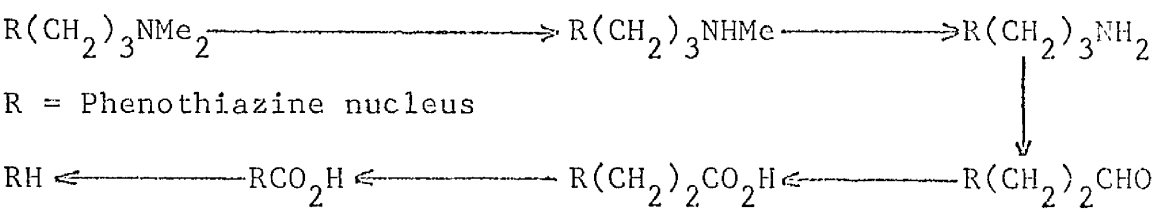
In addition to the above pathways various minor routes of metabolism have been reported from time to time. Initially, there were conflicting views as to whether cleavage of the side chain takes place in such derivatives. Although at first considered probable (Nano and Sancin, 1961), it was quickly discounted by several workers. Berti and Cima (1956) suggested that, since the intense colours found in the urine of phenothiazine dosed animals were not observed

after administration of the tranquillisers, no side chain cleavage could have taken place. Removal of the side chain was also discounted by Walkenstein and Seifter (1959), who observed that although dogs dosed with ^{35}S-phenothiazine excreted 10% of the labelled sulphur in inorganic form, no inorganic sulphate was formed by breakdown of the phenothiazine nucleus after administration of ^{35}S promazine. Similar findings were reported by Fyodorov and Shnol (1956). On the other hand Christensen and Hase (1956) recovered 3-5% of a dose of ^{35}S chlorpromazine from the urine of mice as free inorganic sulphate (23-48% as combined sulphate) indicating that side chain cleavage and subsequent ring degradation had taken place. However, cleavage and subsequent breakdown was thought to be a transformation peculiar to mice.

More recently Beckett et al. (1963) isolated various unidentified non-basic phenothiazine derivatives from human urine after administration of chlorpromazine. They had aldehydic properties, and it was suggested that oxidative deamination had occurred to produce an aldehydic side chain. Since then several metabolites have been reported with properties which would be in keeping with oxidised intermediates of side chain degradation (Posner et al., 1963b; Forrest, Green and Udale, 1964; Green, Forrest, Forrest and Serra, 1965). Forrest et al. suggested that cleavage of the side chain does take place, in several stages, proceeding through demethylation to primary or secondary amines. These are degraded to an aldehyde by oxidative

deamination. This is then oxidised to an acid, which is further converted to a carboxylic acid derivative by β- oxidation. Spontaneous decarboxylation of the latter compound gives rise to phenothiazine. This theory is supported by the identification of 2-chloro-10- (β-propionic acid) - phenothiazine as a metabolite of chlorpromazine in human urine (Rodriguez and Johnson, 1966).

Loss of the side chain, as a biochemical transformation of promazine and chlorpromazine, has now been demonstrated in both man and dog (Fishman and Goldenberg, 1965). Using thin layer chromatography, ultra violet spectroscopy, and comparison with known standards they showed that promazine was degraded to

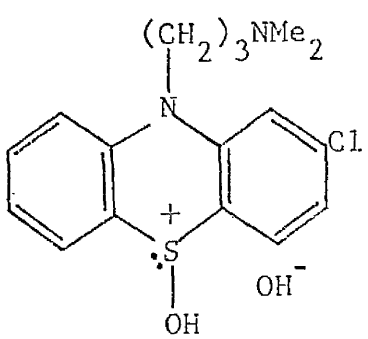


phenothiazine sulphoxide, found both in the free form and conjugated with glucuronic acid. Traces of phenothiazine and phenothiazone were also found. In man the sulphoxide accounted for 0.4% of the dose whereas in the dog 0.8% was excreted. The corresponding chloro-derivatives of these oxidised phenothiazines were obtained from chlorpromazine. Similar results were reported by Johnson, Rodriguez and Burchfield (1965).

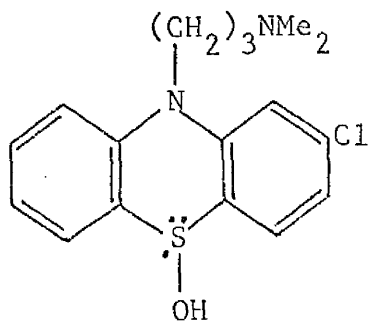
During the development of a rapid urinary colour test for chlorpromazine (Pg. 70), Forrest and co-workers noted that the colour found in positive samples was not produced when chlorpromazine

or its sulphoxide were added to control urines, (Forrest and Forrest, 1957; Forrest, Forrest and Mason, 1958). They postulated that the colour was due to reaction with a metabolite of chlorpromazine with an oxidation level between that of the parent drug and the sulphoxide. Ultra violet irradiation of dilute aqueous solutions of chlorpromazine gave rise to a solution producing the same colour as this metabolite and they claimed that the biochemically and photochemically formed compounds were identical (Forrest, Forrest and Berger, 1958). Two crystalline derivatives were precipitated from the irradiated chlorpromazine solution by addition of excess 2, 4-dinitrophenylhydrazine and sodium nitrite. E.S.R. determinations on those compounds showed that 0.5% of the molecular groups present were in the form of free radicals. From this they postulated that the metabolite was also a free radical and, from spectroscopic considerations, suggested its structure to be the phenazothionium hydroxide (A), in combination with the free radical intermediate (B), rather than the 'thionol' structure (C).

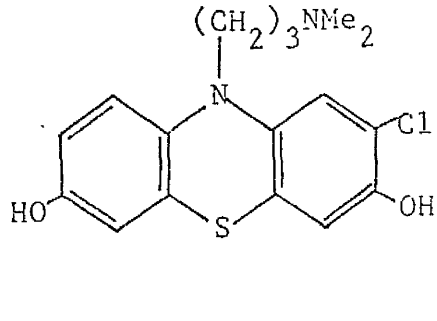
From these investigations it does appear that free radical metabolites of chlorpromazine exist. However, many workers have



(A)



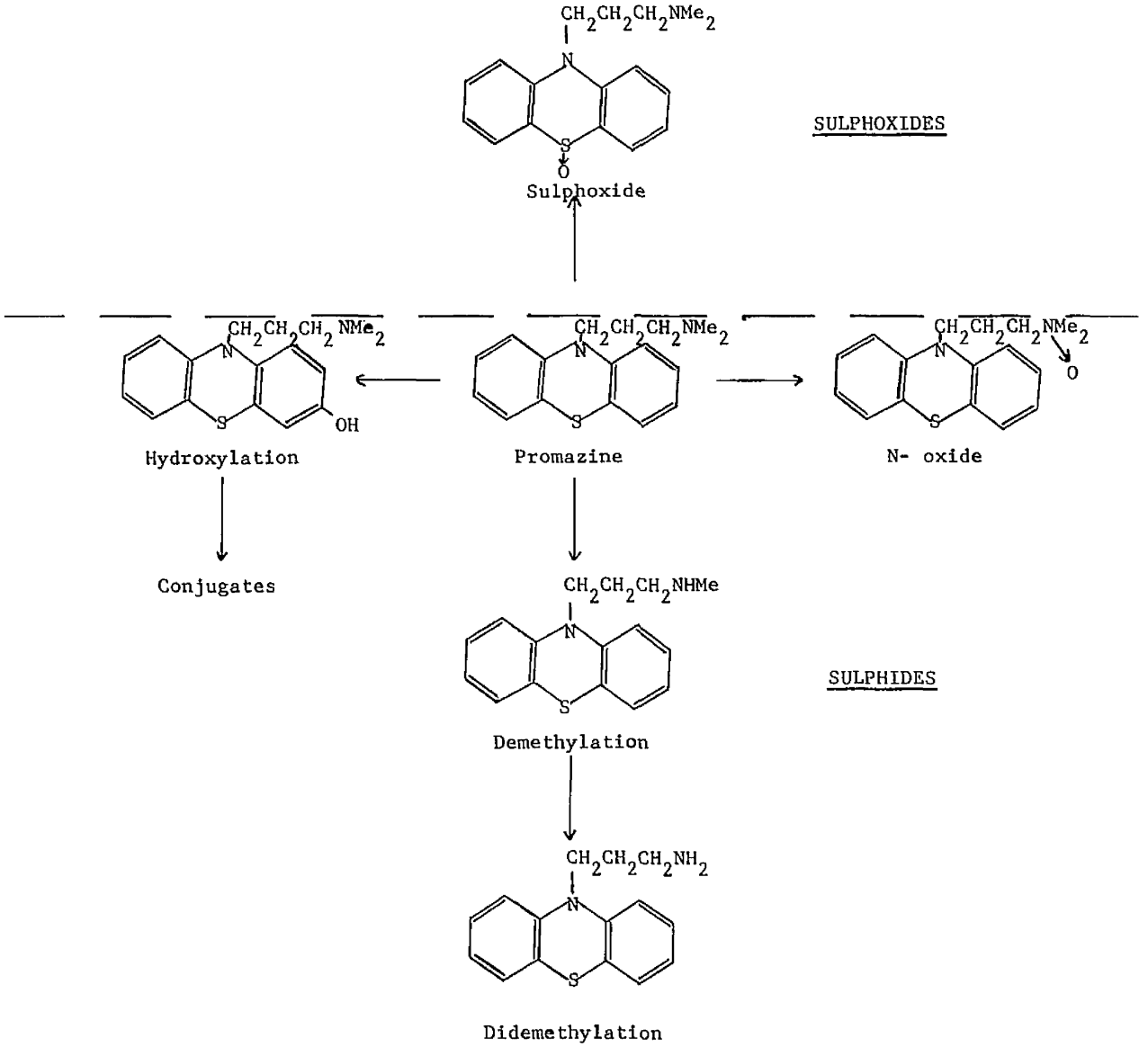
(B)



(C)

FIGURE 6.

METABOLIC PRODUCTS OF PROMAZINE.



shown the diversity in structures of free radical intermediates derived from oxidation of chlorpromazine (Pgs.47-50), and more research is required before the exact nature of such metabolites is elucidated.

The biotransformations of the tranquillisers described can take place either singly or in combination, and a large number of metabolic products are thus possible. The task of determining the specific metabolic products formed by a particular species is further complicated by the slow rates of excretion and low percentage of the dose excreted. Due to these facts the amount of literature on the metabolism and excretion of the tranquillisers is overwhelming, and it could not be hoped to give a complete account of all the variations reported. The major routes of metabolism, using promazine as an example, are shown in diagrammatic form in Figure 6.

ANALYTICAL PROCEDURES FOR PHENOTHIAZINE DERIVATIVES.

III. ANALYTICAL PROCEDURES FOR PHENOTHIAZINE DERIVATIVES

A. Introduction

To examine drug metabolism both qualitatively and quantitatively, suitable methods must be developed for the isolation of the drug and its metabolites from biological material, and for their subsequent separation, identification and quantitation. Many methods have been employed for the detection and assay of phenothiazine derivatives based on their marked reactivity and tendency to form intensely coloured reaction products. However, the application of such techniques to the quantitation of metabolites is a much more difficult problem since in most cases the exact physical, chemical and structural relationship of such derivatives to the parent drug is unknown.

In addition, there is the possibility of interference in such studies by contaminants extracted from the biological medium, possessing similar physical or chemical properties to the drug under investigation (Chapter V). For these reasons there is no completely satisfactory method for the determination of phenothiazine derivatives and their metabolites in biological samples. This section reviews the different assay procedures which have been used for the tranquillisers, and the techniques developed for the isolation, characterisation and determination of their metabolites.

B. Assay of Phenothiazine Tranquillisers.

(1) Colorimetric procedures.

The most common method of analysis is a colorimetric estimation after reaction with an oxidising agent. Eddey and De Eds (1937) determined phenothiazine in pesticide residues from the intensity of the red intermediate, believed to be 3,9-dihydroxy phenothiazonium bromide, formed on reaction with bromine water. The reaction was later investigated by Cupoles (1942) and Fossoul (1950b), and bromination is at present the method of assay for phenothiazine advocated by the Association of Official Analytical Chemists (A.O.A.C., 1965).

Dubost and Pascal (1953, 1955) determined chlorpromazine in biological materials by oxidising with concentrated sulphuric acid, and measuring the intensity of the red product. This procedure was subsequently used by Kok (1955), Berti and Cima (1956) and Thieme (1956). Hetzel (1961) assayed a series of phenothiazine derivatives in biological fluids from the intensity of the yellow products obtained by reaction with concentrated nitric acid. This reagent had previously been investigated by Fossoul (1950a). Promethazine has been determined colorimetrically after oxidation with sodium persulphate (Fossoul, 1951). Chlorpromazine has been determined using an iodic acid-phosphoric acid mixture (Calo et al., 1957, 1958), or peroxide in the presence of peroxidase and catalase (Cavanaugh, 1957). Other oxidising agents which have been employed are bromate (Sandri, 1955), ferric nitrate (Leach and Crimmin, 1956), ferric chloride (Fels and Kaufmann, 1959) and

perchloric acid (Kleinsorge and Thalmann, 1959).

However, the specificity of such reactions has not been described, and, in most cases, either the reagents used or the colours formed are unstable (Hetzel, 1961). Also most of these reactions, although suitable for assay of the larger amounts of material available in pharmaceutical preparations, would be insensitive to the small concentrations of phenothiazines excreted in biological material. Precise estimations are further complicated by the uncertainty regarding amounts of metabolites recovered and their intensity of colour formation compared to the parent drug . Of the reactions described, that using sulphuric acid is the most sensitive and employs stable reagents, although colours tend to fade with time unless a reducing agent is added (Dubost and Pascal, 1955).

Other colorimetric methods of assay have been developed using complexing agents. Overholser and Yoe (1942) used the reaction between phenothiazine and palladous chloride for colorimetric determination of phenothiazine. The method was later used by Cavatorta (1959) as a general assay procedure for promazine, chlorpromazine, and promethazine. By formation of the lauryl sulphate salts of such complexes, Ryan (1959) made the reaction the basis of a general assay procedure for unoxidised phenothiazines.

A further procedure was initially described by Brodie and Udenfriend (1945). It depends on the ability of organic bases to form complexes with sulphonic acid indicators, e.g. methyl orange.

The amount of base is determined by measuring the optical density of the complex in solution. The method has been applied to the assay of diethazine,(Porter and Silber, 1950), and promethazine (Haley and Bassin, 1951).

Nadeau and Sobolewski (1959a, b) determined chlorpromazine and levomepromazine in biological materials from the intensity of their coloured complexes with potassium iodoplatinate. Forrest and co-workers, developed a series of rapid semiquantitative colour tests for phenothiazine derivatives in urine. These tests are based on a combined oxidation-complexing process in which an inorganic ion reacts with a free radical metabolite of the parent drug (Pg. 63). They were reviewed by Forrest et al. (1961).

Drug determination using complexing reagents, however, is subject to the same limitations as the oxidation reactions, (Pg. 69). There is also the possibility using Brodie's sulphonic acid method of reaction with natural organic bases in biological materials in addition to the drug under investigation.

(2) Gravimetric, Volumetric and Electrochemical procedures.

Various volumetric and gravimetric procedures have also been used for the determination of phenothiazine derivatives. Gravimetric methods have included formation of picrates (Uyeo and Oishi, 1952), reineckates (Brauniger and Hoffman, 1955) and silico tungstates (Blazek and Stejskal, 1956). Reineckate and picrate

derivatives were also used by Yung and Pernarowski (1963) in the identification and differentiation of a series of 13 tranquillisers. Other precipitation and crystallisation reactions have been described by Auterhoff (1952), Haas (1952), Haley and Keenan (1950) and Idson (1950).

Volumetric methods have included non-aqueous titration of the dimethylamino group with perchloric acid. Kleckner and Osol (1952) determined promethazine, and Milne and Chatten (1957) determined promazine and chlorpromazine by the method. It was later extended to the assay of several other phenothiazine derivatives in pharmaceutical preparations (Milne, 1959). Non-aqueous titration of the sodium tetraphenyl borates of chlorpromazine and diethazine, and argentometric titration of their hydrochlorides were described by Brauniger and Hoffman (1955).

Several electrochemical methods of analysis have also been developed for phenothiazines in pharmaceutical preparations. An amperometric method of determination was employed by Blazek (1956), and Kabasakalian and McGlotten (1959) used anodic oxidation half-wave potentials for determination of a series of phenothiazine derivatives. Polarographic methods have also been used by Chuen and Riedel (1961) and Porter (1964) who determined chlorpromazine after oxidation with bromine water.

However all three types of procedure (gravimetric, volumetric, electrochemical) require a high degree of purity in the samples under investigation and hence are of little use for the analysis

of biological extracts. Also, as with colorimetric methods, effects due to differences in physical and chemical properties between the parent drug and its metabolites are unpredictable.

(3) Spectroscopic procedures.

Ultra violet spectroscopy has been described for the determination of chlorpromazine, mepazine and promethazine (Meyer, 1957). It has also been widely used for determination of phenothiazine derivatives in biological extracts (Pgs.74-77). This method is much more specific and sensitive than colorimetry and sulphide and sulphoxide derivatives can be differentiated. Also all phenothiazine derivatives have extinction coefficients of the same order (30,000-40,000) which means that metabolites make approximately the same contribution to absorbance as the parent drug. The ultra violet characteristics of phenothiazine drugs are reviewed on Pg. 104.

Fluorimetry has more recently been used by Ragland and co-workers for determination of phenothiazine derivatives in biological materials (Ragland et al., 1964, 1965). It has also been used for the identification of phenothiazine tranquillisers after reaction with potassium permanganate (Mellinger and Keeler, 1963). However, fluorimetry, due to its high sensitivity and low specificity, requires rigid purification not only of the biological extracts but also of all reagents and apparatus.

(4) Radioactive Labelling.

Radioactive labelling has been widely used for the determination

of phenothiazine drugs in biological material. The drug under investigation is labelled at the ring sulphur atom using ^{35}S. Extracts are usually prepared for counting by severe oxidation to form inorganic sulphate, a scintillant added, and the radioactivity measured by a scintillation counter. The absolute activity of the derivatives is calculated by the use of internal reference standards, e.g. $H_2{}^{35}SO_4$, and is expressed as a percentage of the activity originally administered. This provides a value for the percentage of dose excreted which can readily be converted to the concentration or amount of metabolites in the sample.

The technique was first used for phenothiazine derivatives by Christensen and co-workers in a study of the distribution of ^{35}S-chlorpromazine in rat and mouse tissues (Christensen and Wase, 1956; Wase, Christensen and Polley, 1956). The distribution and excretion of ^{35}S chlorpromazine, promazine and chlormepazine has been reported in several species after different routes of administration (Fyodorov 1958). He also reviewed work on ^{35}S phenothiazine derivatives over the period 1955-57. Walkenstein and Seifter (1959) studied the metabolism distribution and excretion of ^{35}S promazine in dogs and rats, and the metabolism and excretion of ^{35}S chlorpromazine by rats was reported by Flanagan et al. (1962) and Emmerson and Miya (1962). Other studies have also been carried out using ^{35}S thioridazine and ^{35}S thiethylperazine (Eiduson et al., 1961, 1963; Zehnder, Kalberer and Rutschmann, 1962).

This labelling procedure is very sensitive and specific to the drug under investigation and metabolites present in equal

amounts make equal contributions to the overall activity. However, the method is expensive both in the cost of the counting equipment and of preparation of the drugs. Also metabolites cannot be differentiated from the parent compound unless the method is carried out in combination with suitable separation techniques (Pg. 79

C. Ultra Violet and Colorimetric Procedures for the Determination of Metabolites in Biological Materials.

Most methods used for the quantitation of phenothiazine derivatives in biological materials involve extraction from an alkaline medium using an organic solvent, and subsequent extraction into acid solution. Concentrations are then measured from the ultra violet spectrum of this acidic extract or from its visible spectrum after suitable colour development.

Berti and Cima (1956) determined chlorpromazine and its metabolites in biological fluids by passing the fluid through an Amberlite IRC-50 ion exchange column on to which the basic phenothiazine derivatives were adsorbed. They then eluted the column with concentrated sulphuric acid and calculated the concentration of phenothiazine derivatives in the eluate from the intensity of the red colour produced. This method, however, is non-selective and groups of metabolites cannot be differentiated. Also, the procedure for colour formation must be standardised to give reproducible results.

Salzman and Brodie (1956) reported a more selective method of analysis. Both sulphide and sulphoxide derivatives were

extracted from the alkaline biological fluid by heptane containing 1.5% isoamyl alcohol. Sulphoxides were then extracted from the heptane into a pH 5.6 acetate buffer, and the sulphides were subsequently taken into 0.1N hydrochloric acid. The amounts of sulphide and sulphoxide were measured separately by ultra violet spectroscopy of the appropriate extract.

Another selective method of assay was reported by Flanagan et al. (1959). They assayed both unconjugated and conjugated metabolites as sulphides and sulphoxides. Unconjugated sulphide and sulphoxide derivatives were extracted from the alkaline biological material by ether then re-extracted into N sulphuric acid. Conjugated metabolites were hydrolysed by heating the residual material in a strongly alkaline medium on a boiling water bath for 1 hour, then extracted in the same way as the unconjugated fraction. The sulphide and sulphoxide contents of each fraction were then determined from the ultra violet spectrum of the appropriate extract. The method of calculation involved a simple background cancellation.

This method, using a single ultra violet spectrum to calculate both sulphide and sulphoxide content, does away with the need for complicated differential extraction procedures. It was subsequently employed by Bolt (1965) for determination of promazine and chlorpromazine, using ethylene dichloride as the extracting solvent, and taking the metabolites into 0.1N hydrochloric acid. He determined the sulphide and sulphoxide content of the glucuronide, sulphate and unconjugated fractions by specific enzyme incubation techniques.

A colorimetric method involving complex formation with potassium iodoplatinate was developed by Nadeau and Sobolewski (1959) The unconjugated fraction, and conjugated fractions after hydrolysis, were extracted into isobutanol and a pH3 buffer solution added. The isobutanol was then evaporated off, thus transferring the metabolites to the aqueous layer. Addition of potassium iodoplatinat and a small amount of gum ghatti to prevent precipitation of the complex, gave an intensely coloured solution. The concentration of metabolites in the sample was measured by comparing the optical density of this solution with calibration graphs obtained using the same reaction with the parent drug. This method, as with the other colorimetric procedures, requires strict standardisation of manipulations for reproducible colour formation. It is also less sensitive than the ultra violet technique, the colour tends to be unstable, and the procedure is not specific for phenothiazine drugs.

The methyl orange procedure of Brodie and co-workers (Pg. 69) was used by Goldenberg and Fishman (1961) to determine unconjugated metabolites of chlorpromazine after elution from paper chromatograms. However this method again is not specific for tranquillisers, and any other organic bases in a urine sample would also form complexes. Some form of purification, e.g. chromatography, is thus necessary before this method can be applied to biological extracts with any accuracy.

Of the methods described ultra violet spectroscopy was the most sensitive, specific, and least complicated procedure. The

method of Flanagan also did not require differential extraction procedures for sulphide and sulphoxide derivatives, their concentrations being calculated from a single spectral curve. It was thus decided to use this method of determination, with modifications for analysis of horse urine (Pg.109). Visible spectroscopy after colour formation with sulphuric acid was also used to corroborate results from the ultra violet spectra.

D. Isolation and Extraction of Metabolites.

Unconjugated metabolites are readily extractable from an alkaline biological medium by organic solvents. On the other hand conjugated metabolites tend to be insoluble in organic solvents, but soluble in water. Such derivatives are therefore subjected to either enzymic or chemical hydrolysis and the liberated phenothiazine fraction then extracted by organic solvents.

Due to the low levels of phenothiazine derivatives excreted in urine, large volumes are needed for the isolation of such compounds, although only small volumes are needed for chromatography. Salzman and Brodie (1956) carried out a large scale ether extraction of chlorpromazine metabolites from dog urine and obtained enough material to identify the sulphoxide. Ether has also been used for the extraction of promazine (Walkenstein and Seifter, 1959) and methoxypromazine (Allgen et al., 1959). Goldenberg and co-workers used ethylene dichloride for the extraction of metabolites of promazine, chlorpromazine and imipramine from human urine (Fishman and Goldenberg, 1962; Goldenberg et al., 1964). They claimed that it was superior to ether in the extraction of sulphoxide

metabolites. Salzman and Brodie (1956) used n-heptane containing 1.5% isoamyl alcohol for quantitative determination of chlorpromazine and its sulphoxide. The same system was used by Allgén et al. (1963) for the extraction of metabolites of levomepromazine. Other solvents used have included isobutanol (Nadeau and Sobolewski, 1959) and chloroform (Kotionis, 1961).

In addition to extraction procedures, adsorption and precipitation techniques have been used for the isolation of glucuronide conjugates especially when large volumes of urine were available. Kamil, Smith and Williams (1952) precipitated phenol glucuronides from rabbit urine by the addition of basic lead acetate, but the method is only applicable to non-basic glucuronides when present in large concentration. Glucuronide metabolites of morphine in human urine have been isolated by adsorption onto charcoal and subsequent elution with acetic acid. (Fujimoto and Way, 1958). On saturation with potassium carbonate a reddish brown gum precipitated from the urine which was dissolved in water and treated with charcoal. A continuous extraction technique using butanol has been employed for the isolation of glucuronides of chlorpromazine and promazine (Beaven, 1962; Bolt, 1965). The butanol extracts were pooled then evaporated under reduced pressure to one fifth of their original volume, when the glucuronides precipitated out. The precipitate was filtered off, washed, and further purified by adsorption and desorption from charcoal.

Such precipitation reactions yield the glucuronides in a very crude form. It was found in preliminary experiments on horse urine, using the technique of Beaven, that precipitates could not be

purified to a sufficient extent to allow identification of the glucuronides. The method of recovery adopted, therefore, was extraction with ethylene dichloride as recommended by Goldenberg and co-workers, and hydrolysis of conjugates using specific enzymes (Pg. 101).

E. Separation Techniques.

Several techniques have been used for the separation and pufification of individual metabolites. Due to the large number of biotransformations undergone by the tranquillisers such techniques must be as sensitive as possible to small differences in structure.

(1) Paper Chromatography.

Paper chromatography was widely used for "phenothiazines" between 1950 and 1960, but has now been largely replaced by thin layer chromatography which is not so time consuming, is more sensitive and gives much better resolution. The latter method, in which the thickness of the layer can be altered, has proved invaluable for preparative work.

Descending paper chromatography of the tranquillisers has most often been used (Allgén et al., 1959; Lin et al., 1959; Fishman and Goldenberg, 1960; Beckettet al., 1963), although ascending techniques (Salzman and Brodie, 1956) and circular development have also been reported (Eisdorfer and Ellenbogen, 1960; Beaven, 1962).

(2) Paper Electrophoresis.

There are very few reports of the use of electrophoresis for purification and separation of "phenothiazine" metabolites. In

their initial studies of chlorpromazine metabolism, Fishman and Goldenberg (1960) separated unconjugated urinary metabolites into three zones by paper strip electrophoresis using 1% potassium carbonate as solvent, but they could not achieve further resolution by variations of pH, etc. Paper electrophoresis was also used by Beaven (1962) and Bolt (1965) for the separation and purification of crude glucuronide metabolites. The latter worker also used continuous electrophoresis over several days. However, this method is time consuming and gives no better resolution than thin layer chromatography.

(3) Thin Layer Chromatography.

Thin layer chromatography has only been applied recently to the separation of "phenothiazine" metabolites. Using this method development is rapid and resolution is high. The resolution is best illustrated by the work of Goldenberg and co-workers who, using this technique, separated and identified over 30 metabolites of promazine and chlorpromazine in the urine of man and dog. This was achieved by developing 250μ thick silica layers in two dimensions For unconjugated metabolites the most suitablesystems were found to be Chloroform-Acetone-Diethylamine (2:7:1) and (88:2:10). The separation of hydroxylated metabolites and hydrolysed conjugates was achieved using Ethyl Acetate-Acetone-Methanol-Diethylamine (68:2:20:15) and Acetone-Isopropanol-1% Ammonia (9:7:4).

(4) Ion Exchange Techniques.

Ion exchange procedures have been used more for isolation of pure metabolic fractions than for separation of individual

metabolites. This is done either by selective adsorption of the phenothiazine derivatives or of the impurities in the biological material, depending on the resin used.

Berti and Cima (1956) isolated the "chlorpromazine"content from urine samples by selective adsorption on Amberlite IRC-50 resin. Eiduson and Wallace (1958) removed 95% of the phenothiazine content from urine samples using this resin. Other cation exchange resins were used by Forrest et al. (1961). They employed Amberlite CG50 for isolation of unconjugated solvent extractable metabolites, and the sulphonated styrene resin, Dowex AG50, for isolation of the total phenothiazine content. Forrest et al. (1961) also used the anion exchange resin, Dowex AG3X4, for removal of indican and other contaminants from urine samples. Lin et al. (1959) isolated small amounts of chlorpromazine metabolites from human urine using Dowex AG50 resin. They also partially separated the metabolites using gradient elution with increasingly concentrated ammonia solutions.

However large volumes of biological material are required for isolation of appreciable amounts of metabolites, and most resins are not specific to the phenothiazines, adsorbing other naturally occurring amines. The adsorption can also be influenced by endogenous electrolytes in the urine.

(5) Gas Liquid Chromatography.

Like thin layer chromatography, this technique has only recently been applied to the separation of phenothiazine derivatives.

Several different stationary phases and detectors have been investigated, and the optimum temperature range for separation has been found to be 200-250°C. Most experiments so far have been on standard reference compounds, and little work has been reported on extracts of urinary metabolites.

Anders and Mannering (1962) chromatographed a series of phenothiazine derivatives using glass columns containing SE-30, a methyl silicone polymer, as the stationary phase. Most of the compounds were readily separated, but those containing piperazine rings were found difficult to elute. In the same year similar separations were reported using different concentrations of SE-30 (Masuda and Ikegawa, 1962; Parker, Fontan and Kirk, 1962; Vanden Heuvel, Haahti and Horning, 1962). A series of 19 phenothiazines have been identified, using silicone oil as the stationary phase, from the gas chromatographic patterns of their pyrolysis products (Fontan, Jain and Kirk, 1964). Bramlett (1965) chromatographed several tranquillisers on Apiezon L claiming that it produced less tailing of peaks than SE-30. All of these techniques used hydrogen flame or β - ionisation detectors.

Gas liquid chromatography of the tranquillisers has been extensively studied by Gudzinowicz and co-workers. Martin, Driscoll and Gudzinowicz (1963) reported a method of calculating their relative retention times, by relating them to boiling point numbers calculated by theoretical chemistry. In the following year they developed a gas chromatographic method for quantitation of chlorpromazine metabolites from human urine after elaborate differential extraction procedures (Driscoll, Martin and Gudzinowicz,

1964). Using this procedure they detected chlorpromazine, its demethylated forms and corresponding sulphoxides down to concentrations of 0.6 mg/litre. They also reported thermal breakdown of sulphoxide and N-oxides, and further investigations showed the main product of decomposition to be 2-chloro phenothiazine. (Gudzinowicz, Martin and Driscoll, 1964). Gudzinowicz (1966) also compared the sensitivity of the hydrogen flame and electron affinity detectors to such compounds. He found that the sensitivity of the latter depended on the grouping at the 2-position, decreasing in the order $Cl > CF_3 > SCH_3 > H > OCH_3$.

Chromatography of chlorpromazine metabolites from human urine was also investigated by Johnson, Rodriguez and Burchfield (1965). They used a microcoulometer as a detector, (halogen and sulphur titration cells), and with the aid of thin layer chromatography identified 10 metabolites. There was also evidence of decomposition of sulphoxides and N-oxides.

Gas liquid chromatography is the most sensitive separation technique yet employed for the tranquillisers, detecting derivatives down to submicrogram levels. However the technique also requires a high degree of purity in the samples under investigation, which is difficult to obtain in biological extracts, (Pg. 258). Column packings and conditions are also difficult to reproduce and decomposition has been shown to occur, especially with metal columns. The applicability of the technique to the separation of phenothiazine derivatives excreted by the horse has been investigated (Pgs. 241-260).

F. Location and Identification of metabolites.

After extraction and separation of individual metabolites, several techniques may be used for their location and identification. These include ultra violet fluorescence, specific spray reagents, comparison with known standards, and elution followed by spectroscopy In the case of gas liquid chromatography, direct entry of the effluent into a mass spectrometer has recently proved a useful identification technique (Pg. 244).

(1) Ultra Violet and Specific Spray Reagents.

Due to their fluorescent properties phenothiazine derivatives can be visualised under ultra violet light. Sulphides produce greenish blue fluorescent spots, sulphoxides appear deep blue, and sulphones pale blue, (Eisdorfer and Ellenbogen, 1960). The sensitivity of the technique is 1-2 µg and it has been widely used for initial location of metabolites after chromatography.

Several general spray reagents have been used for the detection of such compounds. 50% sulphuric acid was used by Salzman and Brodie (1956) for the detection of chlorpromazine and its metabolites. It produces reddish pink colours with sulphides and sulphoxides, and a purple reaction with hydroxylated derivatives. Its sensitivity is approximately 1 µg.

Another general reagent consists of a few crystals of sodium nitrate dissolved in 10ml concentrated hydrochloric acid (Sprogis et al., 1957). It produces a variety of colours with sulphides and sulphoxides but does not react with sulphones. Its sensitivity is also of the order of 1 µg. The Munier iodoplatinate reagent (Munier and Machebouf, 1949) has also been used for the detection

of "phenothiazines" (Eidorfer and Ellenbogen, 1960). However the spray is not specific for the phenothiazine nucleus but reacts with the side chain amine group of the molecule. It consists of 5ml 5% w/v aqueous platinic chloride, 45ml 10% w/v aqueous potassium iodide, and 50ml water. Its sensitivity has not been described.

Fishman and Goldenberg (1960) used ammonium persulphate as a general reagent. It reacted with sulphides and sulphoxides producing a variety of colours, but not with sulphones. Its sensitivity is approximately 2 µg. 3-methyl-2-benzothiazolone hydrazone produces colours with phenothiazine derivatives in the presence of ferric ions (Sawicki et al., 1961 a,b,c). It reacts with sulphides, sulphoxides and sulphones but is not specific for phenothiazine derivatives. The active species is the aniline type grouping within the nucleus, and the mechanism of the reaction is thought to be diazotisation at the para position to the nitrogen. Its sensitivity was not specified.

Several more specific reagents have been developed for detection of structural modifications to the parent compounds. An acidic ferric chloride dip for paper chromatograms was described by Fishman and Goldenberg (1960). It produces a red coloration with sulphides but does not react with sulphoxides or sulphones. The solution consists of 1% w/v ferric chloride in 2.5N nitric acid. Sulphides can be detected down to the 5 µg level using the reagent.

A spray using 1% aqueous sodium metaperiodate, which is specific for hydroxylated phenothiazines, has been described (Bolt,

1965). It produces transient greenish-purple colours with hydroxylated phenothiazines which rapidly change to yellowish-brown. The sensitivity is also approximately 5 µg.

Two sprays have been developed for the detection of demethylation of the terminal amino group which differentiate mono- and didemethylation. Ninhydrin (Indane-1,2,3-trione) reacts with primary amines giving bluish-purple colours, and also has a weak reaction with secondary amines. The reagent consists of a 1% solution of ninhydrin in 10% v/v aqueous acetic acid. After spraying, the plate is heated at 100°C for 10 minutes. Its sensitivity in detecting desdimethyl chlorpromazine was approximately 10 µg. Walkenstein and Seifter (1959) used a nitroprusside reagent for the detection of secondary amines. It consists of two solutions, A and B, which are mixed in equal proportions immediately before use. A is a 3% solution of sodium nitroprusside in 10% aqueous acetaldehyde, and B is a 2% aqueous solution of sodium carbonate. It produces a bluish-purple colour with such derivatives and, using desmonomethyl chlorpromazine, was found to have a sensitivity of 30 µg.

Of the general reagents it was decided to use sulphuric acid in combination with the ultra violet technique for location of metabolites on thin layer plates. The colours produced are specific for phenothiazines, it has high sensitivity and hydroxylated forms can be distinguished. All the more specific sprays described were also used. The method of spraying is described on Pg. 118.

(2) Spectroscopic Identification.

Many workers have investigated the possibility of determining the structure of metabolites by spectroscopic techniques. Ultra

violet spectroscopy readily differentiates sulphides and sulphoxides, and has been used to confirm results using the ferric chloride spray. Several attempts have also been made at determining the nature and the position of substituents from spectra recorded after reaction with sulphuric acid. Rieder (1960) used this method for a series of 2-substituted phenothiazines and related the wavelength of the major peak in the visible region to the nature of the substituent. A similar study was carried out by Street (1962) who based his identification on absorbance wavelengths in the ultra violet region. However such studies produced no informatio regarding the position of substituents. Beckett and co-workers studied ultra violet and visible spectra of a series of methoxy- and hydroxy-phenothiazines after reaction with 50% sulphuric acid and differentiated the 1-, 2-, 3- and 4- derivatives from their absorbance maxima in the 340-380mμ region. (Beckett and Curry, 1963; Beckett, Curry and Bolt, 1964). Thus, provided a concentrated enough sample can be eluted, it should be possible to differentiate monohydroxylated metabolites by this method.

Infra red spectroscopy has also been investigated as a means of determining the nature and position of nuclear substituents. Many reports of the infra red spectra of both mono- and multi-substituted phenothiazines have appeared in the literature (Smith, 1950; Charpentier et al., 1951; Massie, 1954; Roe and Little, 1955; Cymerman-Craig et al., 1956; Yale et al., 1957; Nodiff and Craig, 1961).

Different functional groups can be recognised by this method

e.g. hydroxylation, from the O-H stretching frequency, and demethylation, from the N-H stretching frequency. However, due to their proximity, care should be taken not to confuse these two frequencies. Sulphoxide derivatives have also been reported to give a strong peak at 1020 cm^{-1} due to the S-O stretching vibrations (Patterson et al., 1954). However, due to the complexity of such spectra the method should be used more as confirmation of other methods, e.g. spray reagents, ultra violet spectroscopy.

Attempts have also been made to determine the position of substitution from such spectra. Nodiff and Craig (1961) examined the C-H out of plane deformation modes of the benzene rings and reported that 2- or 3- substituted derivatives have a strong peak between 800 and 550 cm^{-1} similar to an asymmetric trisubstituted benzene. 1- and 4- substituted derivatives on the other hand have a strong peak between 750 and 800 cm^{-1} corresponding to a vicinally substituted benzene.

Bolt (1965) studied a large series of substituted phenothiazines in three regions of the spectrum corresponding to the aromatic C-H deformation, the C-C skeletal, and the O-H stretch modes. He concluded that in the 700-900 cm^{-1} range, (C-H deformation modes), it is possible to tell whether one or both rings is unsubstituted or monosubstituted. Also, if monosubstitution occurs, it is possible to tell the position. With more than one substituent in a ring no assignment can be made. The C-C skeletal mode is of no use in determining the position of substitution. The O-H stretching frequency can be used to a certain extent to give positions of

hydroxylation but should not be confused with the N-H stretch of primary or secondery amines, (Demethylated derivatives).

Such structural assignments are very complicated, and the method is probably of much more use when synthetic reference compounds are available to fingerprint all the vibrational frequencies involved. However, both this technique and ultra violet spectroscopy used in connection with the specific spray reagents can be of some value in confirming structural assignments.

AIMS OF THE INVESTIGATION

There have been few reports regarding metabolism and excretion of the phenothiazine tranquillisers by the horse, although such processes have been widely studied in other species (Chapter II). Carey and Sanford (1963) detected 7 metabolites of promazine in horse urine and found that polar metabolites predominated. Schubert (1967) reported prolonged excretion of chlorpromazine over several days after a single intravenous injection, and that only a small percentage of the dose was excreted. He detected 4 metabolites, the major one being chlorpromazine sulphoxide. Since so little information was available, it was decided to carry out a detailed analysis of metabolism and excretion by the horse of the tranquillisers more commonly used in equine practice. These processes were followed both qualitatively and quantitatively after both oral and parenteral administration.

Interference with analysis of phenothiazine derivatives due to contaminants in horse urine was reported by Schubert (1967), and was noted during preliminary experiments for the present study (Chapter V). Different methods of collection, storage and analysis were therefore investigated in an attempt to eliminate such interference.

Experiments were also carried out to evaluate the applicability of the methods developed to the detection of small doses of phenothiazine derivatives just sufficient to produce an effect. The use of the gas chromatograph-mass spectrometer was also investigated for this purpose.

METHODS, MATERIALS AND APPARATUS.

IV METHODS, MATERIALS AND APPARATUS

A. Urine Collection.

(1) Introduction.

Harnesses for the continuous collection of urine from geldings were described by Lindsey (1926), Howell (1930) and Vander Noot et al. (1965). All incorporated a funnel of canvas or similar material suspended under the penis, and connected to a collection vessel by large bore tubing. Those of Howell and Vander Noot also included equipment for the collection of faeces, comprising a large rubber lined canvas bag strapped firmly to the buttocks of the animal. The former used the further innovation of a lid on the bag, opened before defaecation by the upward movement of the horse's tail. Vander Noot et al. also described a metabolism stall for use in conjunction with their collection apparatus.

More recently a harness was developed using a funnel of reinforced latex (Warwick, 1966), which was modified by Nicholson (1968) by incorporating a paddle arrangement in the tubing between the funnel and the collection vessel. Flow of urine through the tubing depresses the paddle thus closing an electric circuit. This, in turn, operates a pointer on a smoked drum, revolving once a day, to give a record of each urine sample collected.

There are comparatively few reports of urine collection harnesses for mares, probably due to the difficulty in retaining a collection device securely in position. A typical arrangement was described by Grandier (1960) in which a collection bag was

connected to the animal in such a way as to cover the vulvar cleft.

In the various harnesses developed the strapping arrangement seemed much more complicated than need be, and the funnels and tubing were heavy, which would tend to make the equipment cumbersome and annoying to the animal. The fact that the funnel was connected to a fixed reservoir, thus requiring the use of a metabolism stall, or immobility over long periods, would also lead to the annoyance of the animal, and the need for frequent exercising.

(2) Development of a Harness.

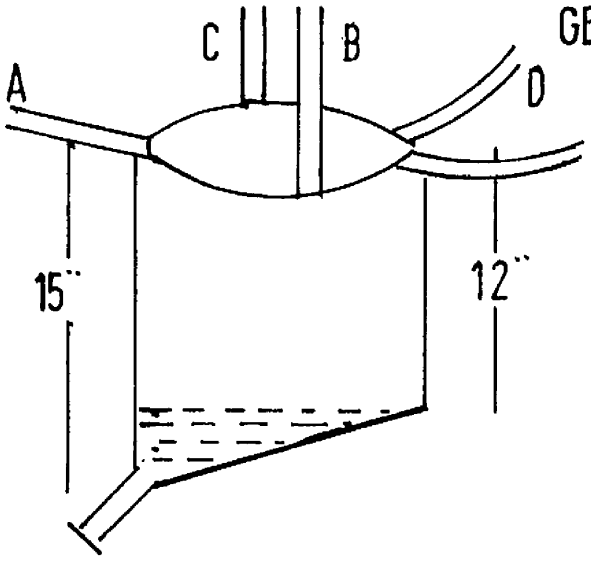
With these points in mind, a harness for the collection of urine from geldings was designed for the present work,* which has the advantages of being light, with the minimum of strapping arrangements, giving maximum freedom to the animals. The arrangement can be assembled and fitted within minutes at a negligible cost, the collection bags are readily interchangeable, and an entire collection can be supervised by one person.

The collection container is a double polythene bag cut in such a way as to leave one of the bottom corners slightly lower than the other (Fig. 7A). A small hole is cut in this lower corner, through which a 3" length of $\frac{1}{4}$" diameter rubber tubing is inserted, and firmly bound by thread, to produce a watertight junction between it and the polythene. A stopper is then fitted to this outlet tube.

* I am grateful to Miss F.M. Carey for her help in the development of this collection harness.

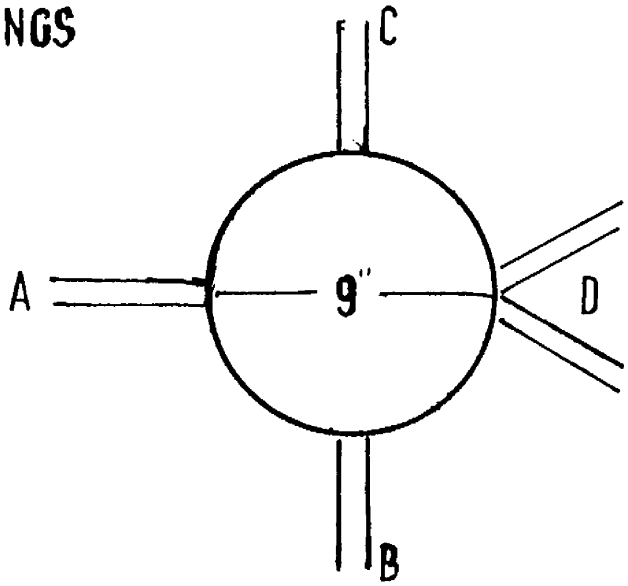
FIGURE 7.

METABOLISM HARNESS FOR CONTINUOUS COLLECTION OF URINE FROM GELDINGS



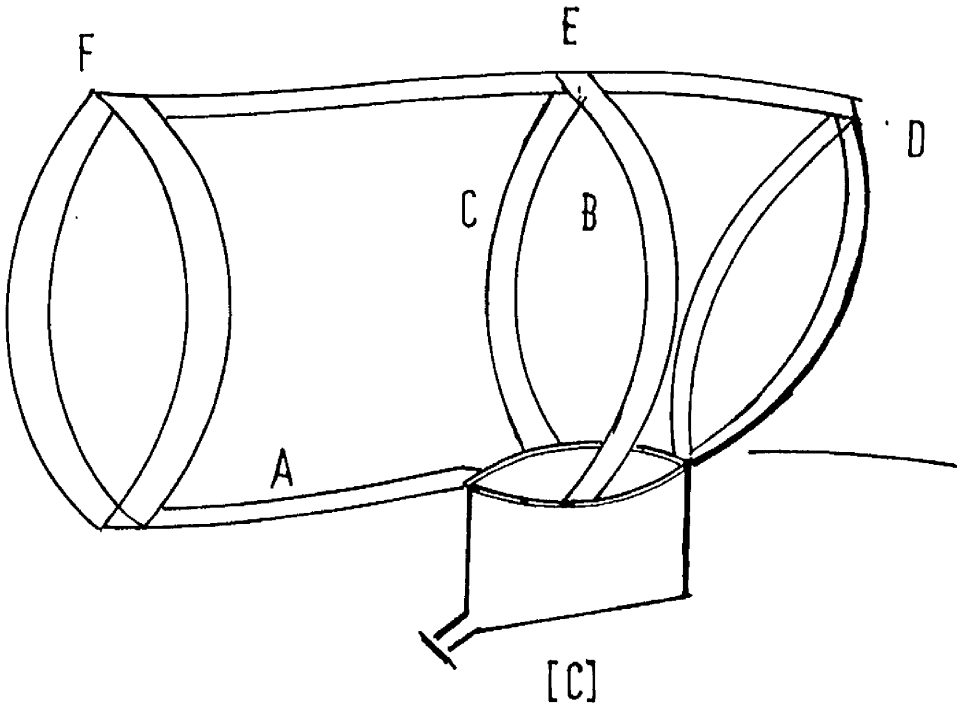
[A]

SECTIONAL VIEW OF COLLECTION BAG.



[B]

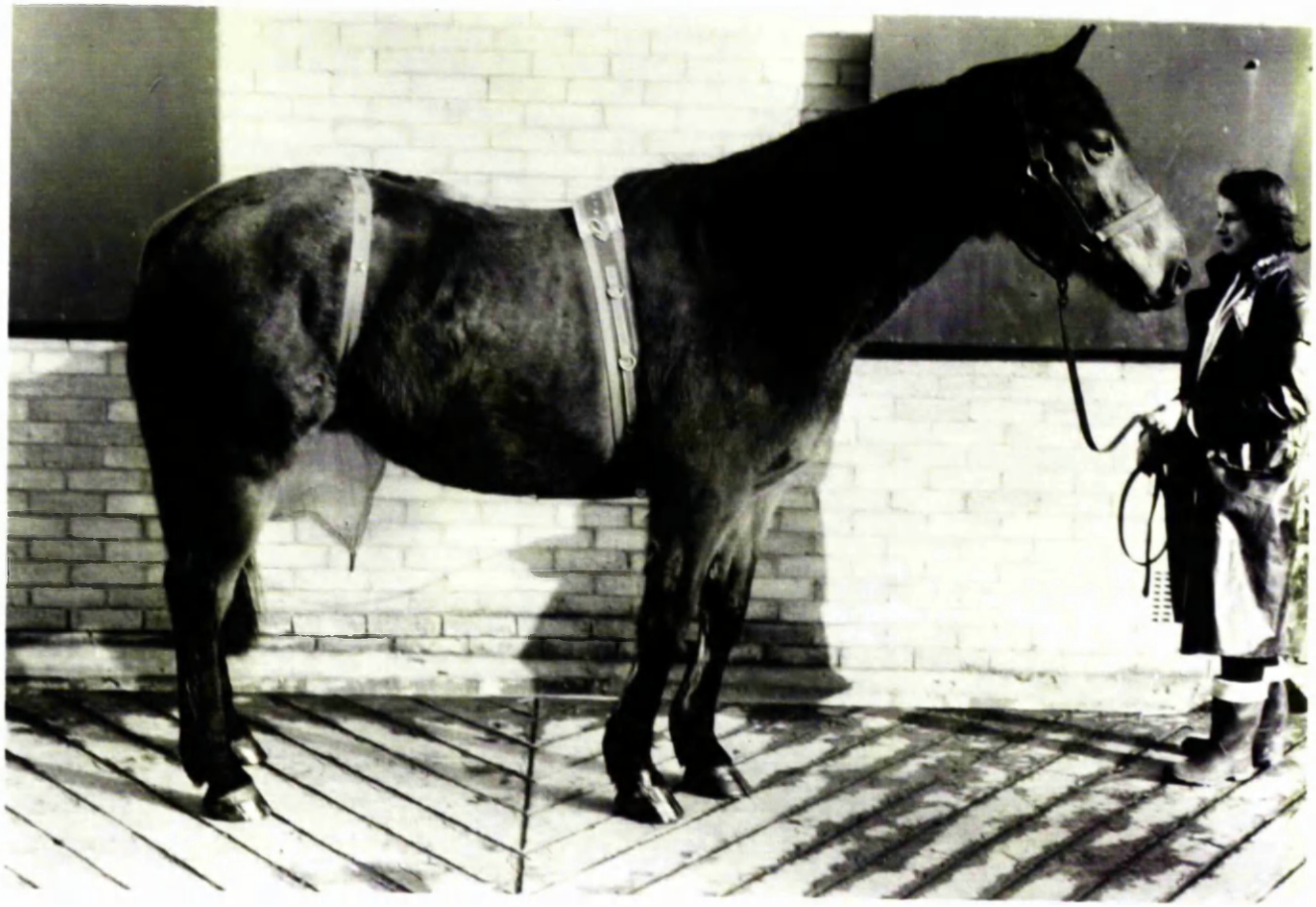
PLAN OF COLLECTION BAG.



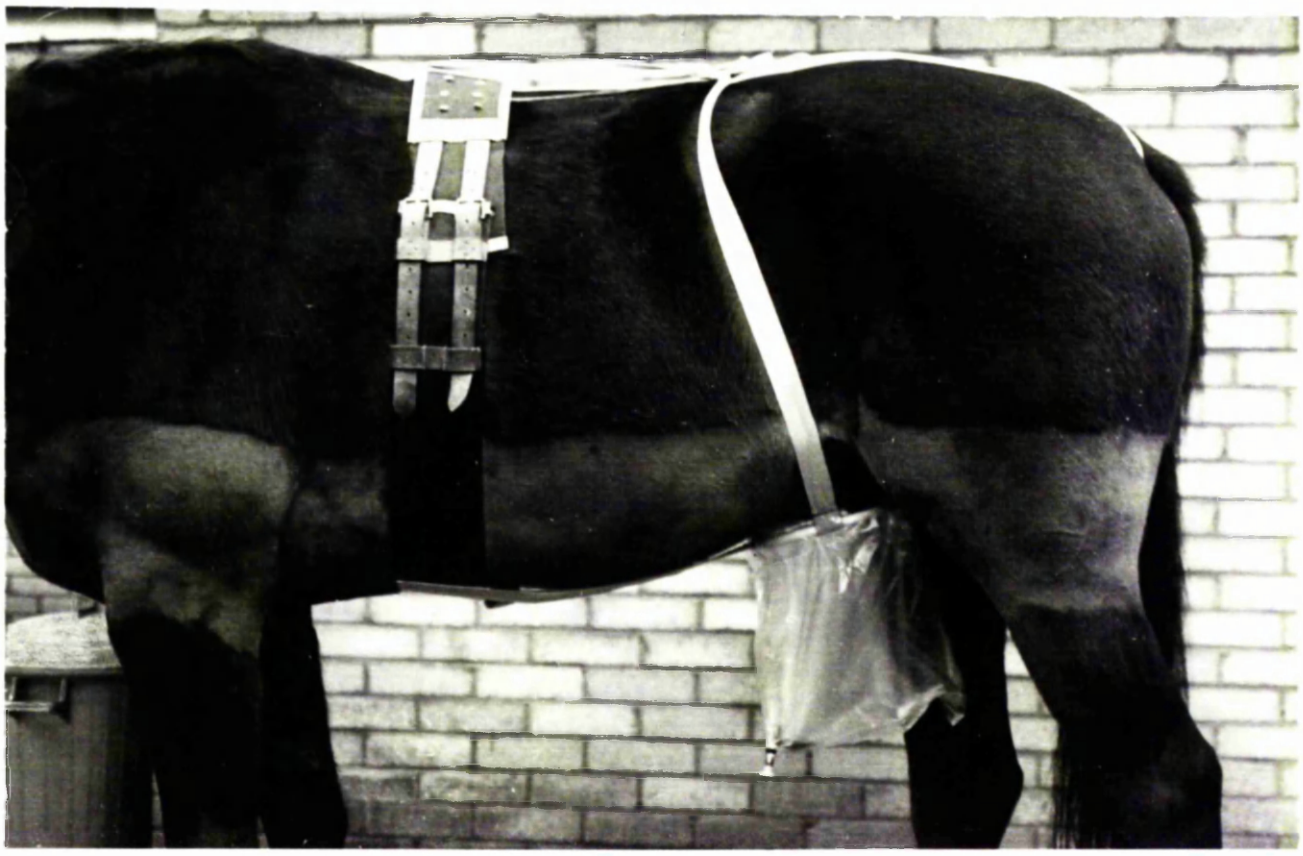
[C]

DIAGRAM OF STRAPPING ARRANGEMENT FOR HARNESS.

FIGURE 8.



METABOLISM HARNESS IN POSITION.



The top of the bag is firmly bound round a 9" hoop of $\frac{1}{2}$" diameter plastic tubing which forms a rigid framework for the mouth of the vessel. Four slits are then cut in the polythene just below this frame, and straps A, B, C and D, of $1\frac{1}{2}$" webbed tape, are attached (Fig. 7B).

To fit the harness, the front strap, A, is drawn through a 5" surcingle already in position, and secured. Straps B and C are then fastened over the horses back at E (Fig. 7C), and the double tape D is pulled between the hind legs, over the tail, and secured at the same position (E). Finally this junction is attached to the surcingle at F by a strap along the back of the animal. Minor adjustments are then made to all straps to ensure that the harness fits comfortably, with the collection bag in the correction position. Figure 8 shows the apparatus fitted to one of the geldings used in the present work, (8).

(3) Automatic Collection.

The harness was further developed with a view to automatic collection of samples. The apparatus used is shown diagramatically in Figure 9. Instead of a stopper, a length of pressure tubing was attached to the outlet from the collection bag, and threaded up the side of the surcingle, where it was held firmly in place. It was then led through a series of elastic supports on the ceiling of the loose box, and through the wall to an aspirator in the adjoining laboratory. The elasticity of the ceiling supports and the length of the pressure tubing were such that the horse had complete freedom of movement throughout the collection.

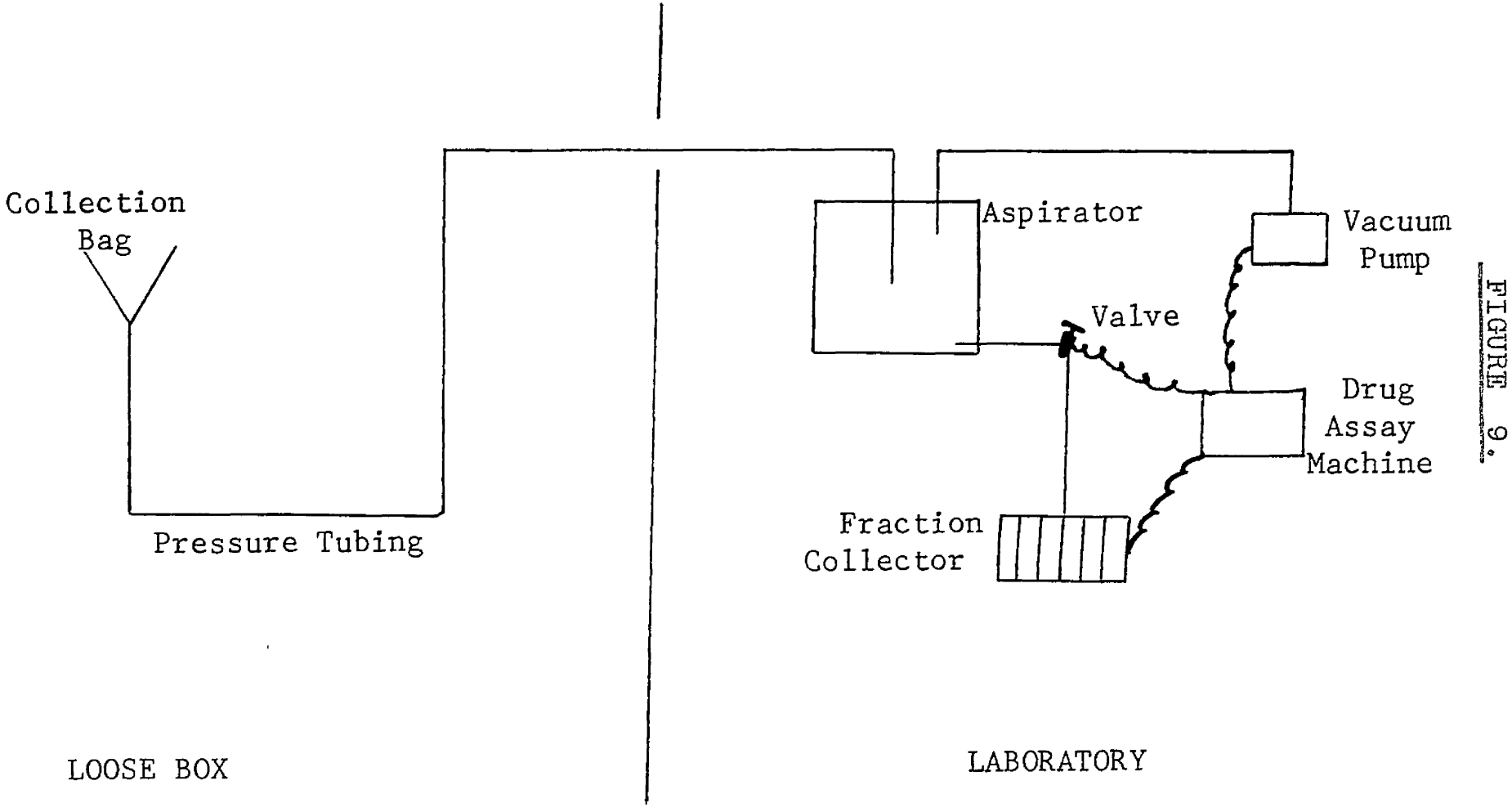


FIGURE 9.

SCHEMATIC DIAGRAM OF APPARATUS FOR AUTOMATIC COLLECTION OF URINE.

The principle is that urine is passed into the bag, connected through the aspirator to a vacuum pump. The pump is switched on at hourly intervals by a signal from an automatic assay machine, removing the urine from the bag to the aspirator. The pump is then switched off and the outlet valve from the aspirator is opened by the assay machine, allowing the sample to flow, by gravity, into a vessel on the fraction collector. Two final signals from the assay machine close the outlet valve and move the fraction collector on to the next position.

The fraction collector was assembled by connecting the outlet tube from the aspirator to the revolving drum of a kymograph, synchronised to bring the tube over flasks set at regular intervals corresponding to its movement at successive collections. The apparatus has been successfully used for collection of urine over periods of up to 48 hours.

(4) Experimental Animals, Sample Collection and Storage.

Details of the horses used, which were either light hunters or thoroughbreds, are given in Table 2. Each horse was brought in from grass at least three days before drug administration. This interval allowed a decrease in urinary output, giving much more manageable and concentrated amounts for extraction purposes than would be obtained from an animal straight from grass. Before and after drug administration they were fed a standard diet of 2 feeds per day. The morning feed (8.00 - 9.00 a.m.) consisted of 2lb. bran and 4 lb. nuts, and the evening feed (4.00 - 5.00 p.m.) of 2lb. bran and 2lb. oats. They also received 20lb. hay morning

TABLE 2. EXPERIMENTAL ANIMALS.

HORSE	NAME	AGE	HEIGHT	WEIGHT	SEX	BREED
1	GERTRUDE	7Yr	15·1Hd	700Kg	♂	BROWN IRISH BRED COB
2	SHANE	AGED	15·1Hd	500Kg	♂	BAY ANGLO ARAB
3	TOR	AGED	15·2Hd	500Kg	♂	DARK BAY THOROUGHBRED
4	KILDARE	AGED	16·1Hd	600Kg	♂	BAY IRISH BRED HUNTER
5	LAD	AGED	15·3Hd	500Kg	♂	BAY THOROUGHBRED
6	OSCAR	6Yr	17 Hd	700Kg	♂	DARK GREY ROAN IRISH BRED HUNTER
7	SQUIFFY	AGED	15·1Hd	500Kg	♂	HALF BRED CHESTNUT ROAN
8	TEDDY	6Yr	16·1Hd	500Kg	♂	BROWN THOROUGHBRED

and evening.

Collections were made from geldings using the harness described, and in the case of mares a catheter was passed at 2 hourly intervals. The latter method was adopted since it was found very difficult to keep a collection bag in position, especially over the prolonged periods involved. An indwelling catheter was also considered impractical due to the temperament of the animals.

All samples were stored, in darkness, in sterile containers at -20°C, since it had been found in preliminary experiments that storage at 4°C allowed a build up of impurities, which tended to mask the phenothiazine peaks on the ultra violet spectra of extracts (Chapter V).

In each experiment a control sample of urine was taken on the first morning, before drug administration, and samples were then collected individually for as long as required. The volume and pH of each sample was measured. Although, initially, individual samples were analysed, it was subsequently found that analysis of pooled samples gave much more uniform results (Pg. 140). 100 ml aliquots from each sample, unless otherwise stated, were used for quantitative analysis by ultra violet spectroscopy, and 300 ml was used to obtain qualitative excretion patterns of individual metabolites by thin layer chromatography. All residual urine was pooled for further structural identification of metabolites.

B. Drug Administration and Dosage.

For the major quantitative and qualitative studies, drugs were administered between 9 and 11 a.m. on the first day of each

experiment. Intramuscular doses were injected into the neck muscles, several sites being chosen to avoid inflammation due to the large volumes involved. For oral dosing, tablets were powdered, mixed with treacle, and incorporated in the morning feed.

Drugs and doses, which were considered to be the maximum which could be safely administered, were:

Chlorpromazine hydrochloride (Largactil - May & Baker).

Intramuscular, as a 5% w/v solution - 2 mg/Kg. Oral, in powder form - 5 mg/Kg.

Promazine hydrochloride (Sparine - Wyeth).

Intramuscular, as a 5% w/v solution -5 mg/Kg. Oral, in powder form 10 mg/Kg.

Acepromazine maleate. (Acetylpromazine - Boots).

Intramuscular, as a 1% w/v solution - 0.5 mg/Kg. Oral, in powder form - 1 mg/Kg.

Propionylpromazine phosphate (Combelen - Bayer)

Intramuscular, as a 1% w/v solution - 0.5 mg/Kg.

In addition to these doses, smaller amounts were administered to ascertain limits of detection (Pgs. 224, 230, 236.), and repeated daily doses were used for studies into length of excretion. After drug administration clinical and physiological effects were recorded until the animal returned to normal (Pg. 138).

C. Materials and Apparatus.

(1) Reference Compounds and Enzyme Preparations.

Chlorpromazine, desmonomethyl chlorpromazine, desmonomethyl promazine, desdimethyl chlorpromazine, chlorpromazine sulphoxide

and chlorpromazine N-oxide were kindly supplied by May & Baker, Ltd., promazine and promazine sulphoxide by John Wyeth and Brothers, and acepromazine and acepromazine sulphoxide by Boots Ltd. I am grateful to Dr. F. Leonard of the National Institute of Mental Health for samples of 3-hydroxy promazine and 7-hydroxy chlorpromazine Ultra violet and mass spectra, colour reactions with the specific spray reagents, and Rf values in several solvent systems were recorded for each compound. β-glucuronidase, (Ketodase 5000 units/ml was supplied by William Warner & Co. Ltd., and sulphatase(Mylase P) by Sigma Ltd.

(2) Instruments.

Ultra violet and visible spectra were recorded on a Beckman DB spectrophotometer. Gas chromatograms were recorded on an Aerograph 1522-B instrument, using flame ionisation detectors. Mass spectra were recorded on the LKB mass spectrometer-gas chromatograph. The thin layer plates were visualised by a Minuvis Ultra violet lamp (Desaga).

D. Quantitative Analysis.

(1) Extraction Procedures.

The methods of extraction employed are summarised in Figure 10. Metabolites were extracted as three fractions - unconjugated, glucuronide conjugated and sulphate conjugated derivatives.

(a) Unconjugated Metabolites - Procedure 1.

100 ml aliquots of urine were adjusted to pH 9.5 - 10 using

EXTRACTION PROCEDURES FOR PHENOTHIAZINES AND THEIR METABOLITES

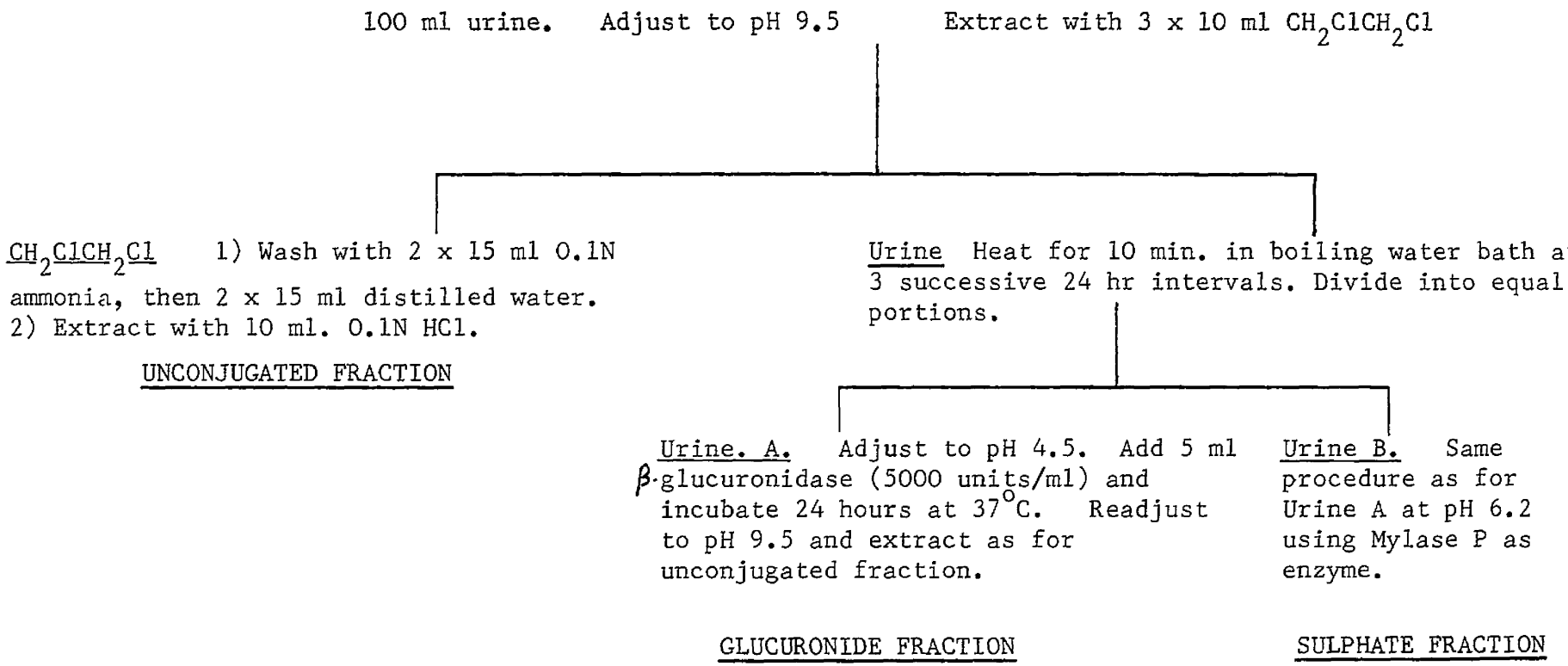


FIGURE 10.

N ammonia solution. The white precipitate which formed was removed by centrifuging, and decanting the supernatant urine. (This precipitate contained no phenothiazine derivatives as shown by reaction with 50% sulphuric acid. Its composition is described in Chapter V (Pg.123).) The urine was then extracted with three 10 ml volumes of 1,2-dichloroethane (previously washed successively with half its volume of 0.1N ammonia solution, 0.1N hydrochloric acid, and distilled water), and the organic extracts were pooled. The residual urine was stored at -20°C for analysis of conjugated metabolites. The organic extract was then washed with 0.1N ammonia (2 x 15 ml) followed by distilled water (2 x 15 ml), and finally extracted into 10 ml 0.1N hydrochloric acid. This solution was assayed for phenothiazine content by ultra violet spectrometry (Pg. 109).

Emulsions formed at any stage of the extractions were usually broken by centrifuging at approximately 2,500 revs./min, and, if this failed, the addition of a little anhydrous ammonium sulphate succeeded.

(b) Conjugated Metabolites - Procedure 2.

As analysis of conjugated metabolites involved prolonged incubation of the residual urine from Procedure 1, it was first sterilised by heating for 10 minutes on a boiling water bath at three successive 24 hour intervals. This avoided the possibility of formation of contaminants by microbial action at 37°C, (Chapter V). Thereafter it was treated under sterile conditions. After sterilisation the urine was divided into two equal portions, one for the analysis of glucuronides, and the other of sulphate

conjugated metabolites.

(i) Procedure 2 (a) - Glucuronide Conjugated Metabolites.

The first portion was adjusted to pH 4.5 using 5N hydrochloric acid, and 5 ml β-glucuronidase (Ketodase ~5,000 units/ml) were added. It was then incubated for 24 hours at 37°C, all glassware required from this point being flamed before use. After incubation the pH was adjusted to 9.5 - 10 using N ammonia solution, and extraction was carried out as for Procedure 1 with proportionate volumes of solvent.

(ii) Procedure 2 (b) - Sulphate Conjugated Metabolites.

The second portion was adjusted to pH 6.2 using 5N hydrochloric acid, and sulphatase (Mylase P) was added in 1% concentration. The urine was then incubated for 24 hours at 37°C, adjusted to pH 9.5 - 10, and extracted by Procedure 1.

(2) Ultra Violet Characteristics of Phenothiazine Derivatives.

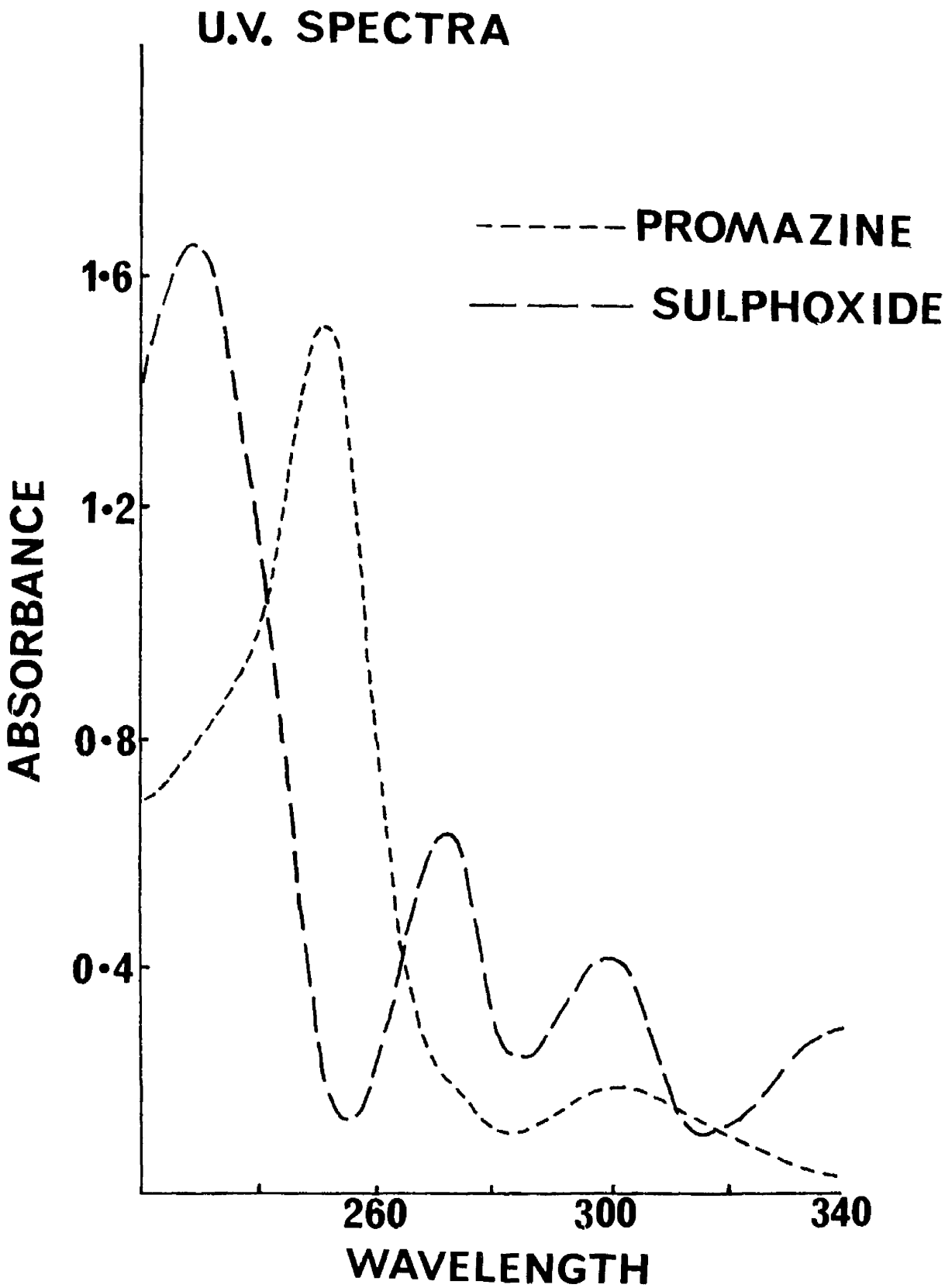
Ultra violet spectra of the phenothiazine derivatives available (Pg. 100) were recorded in acid, neutral and alkaline solution. For acid and neutral conditions, solutions (10 mg/ml) were made up in 0.1N hydrochloric acid and water respectively, and for an alkaline medium a few drops of 0.1N ammonia solution were added to the neutral sample. Visible spectra were also recorded after reaction with 50% sulphuric acid. 2 ml. concentrated sulphuric acid was slowly added dropwise to 2 ml. of each solution (50 mg/l) under constant cooling with running water. Solutions were then allowed to stand 10 minutes for maximum colour development, and their spectra recorded.

The compounds had characteristic ultra violet spectra and high extinction coefficients (30,000 - 40,000). With the exception of acepromazine and propionylpromazine, all sulphide derivatives (i.e. those in which the sulphur atom is unoxidised) had a major peak between 250 and 260 mμ and a minor one between 300 and 320 mμ. These are typical absorbance wavelengths for tricyclic aromatic hydrocarbons (Badger, 1954). In acid solution chlorpromazine had maxima at 255 and 308 mμ, and promazine at 252 and 300 mμ. All sulphide derivatives of each drug, had absorbance maxima within 2 mμ of the parent compound.

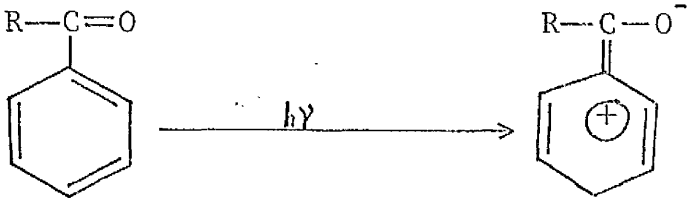
Oxidation to the sulphoxide form produced vast changes in spectral behaviour, four peaks, one major and three minor, being formed instead of two. The major peak appeared between 230 and 240 mμ and the minor ones in decreasing order of intensity at approximately 270, 300 and 340 mμ. In acid solution chlorpromazine sulphoxide had peaks at 240, 274, 302 and 342 mμ and the corresponding wavelengths for promazine sulphoxide were 230, 272, 300 and 340 mμ. Similar spectral differences between sulphide and sulphoxide derivatives have been reported for phenothiazine (Houston et al., 1949), methoxypromazine (Allgen et al., 1959) and mepazine (Hoffman et al., 1959). Further oxidation of such derivatives to the sulphone causes a lowering in wavelengths of all four sulphoxide peaks (Bolt, 1965). Typical spectra of sulphide and sulphoxide derivatives, using promazine as an example, are shown in Figure 11.

As previously stated, acepromazine and propionylpromazine have a different pattern of ultra violet spectra from that just

FIGURE 11.


U.V. SPECTRA
PROMAZINE
SULPHOXIDE
ABSORBANCE
1·6
1·2
0·8
0·4
260
300
340
WAVELENGTH

described. In acid solution the parent drugs produced two peaks of almost equal intensity at approximately 240 and 230 mμ and the sulphoxide derivatives had a major peak at approximately 255 mμ and a minor one at approximately 315 mμ. The ultra violet spectra of acepromazine and its sulphoxide are shown in Figure 12. The characteristic spectra of these two compounds are probably due to the presence of the side chain carbonyl group directly attached to the nucleus (an additional chromophore to the aromatic system of the other phenothiazine derivatives), in conjugation with the aromatic ring. By analogy with ketone substituted benzene rings this would lead to changes in the wavelength and intensity of absorbance of the phenothiazine nucleus due to the electron transfer process

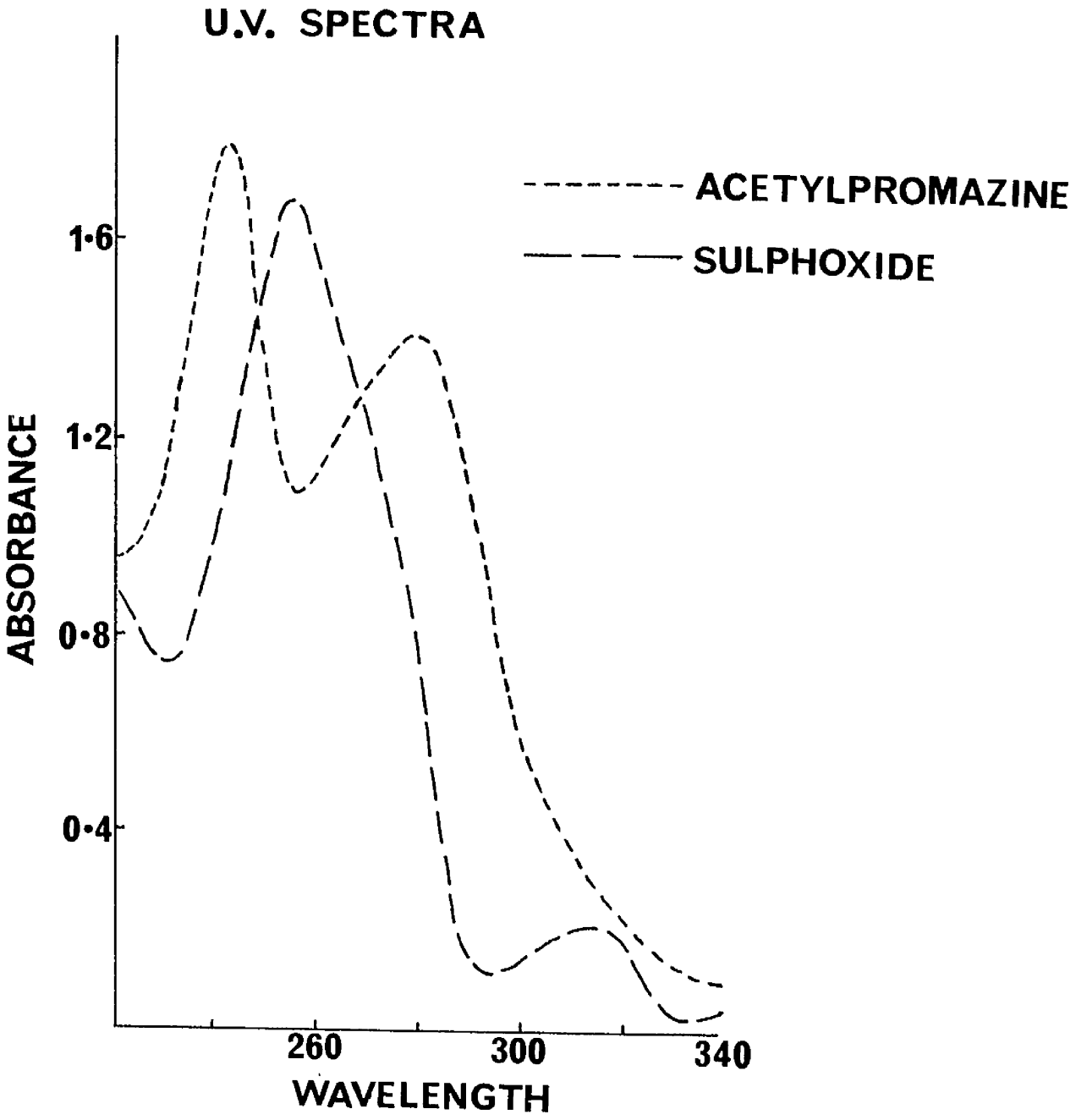


shown (Murrell, 1961; Scott, 1961).

On reaction with 50% sulphuric acid all the derivatives studied had one major absorbance peak in the visible region between 510 and 540 mμ. For all derivatives of promazine and acepromazine, including sulphoxides, this occurred at approximately 515 mμ, whereas for chlorpromazine and its derivatives it occurred at approximately 530 mμ.

Thus phenothiazine derivatives can be divided by their ultra violet spectral properties into two groups - those with spectra resembling that of promazine and those resembling acepromazine. The groups can be further divided from their spectra

FIGURE 12.


U.V. SPECTRA
ACETYLPROMAZINE
SULPHOXIDE
ABSORBANCE
1·6
1·2
0·8
0·4
260
300
340
WAVELENGTH

into sulphide, sulphoxide and sulphone derivatives. Also each drug, on reaction with 50% sulphuric acid, has a characteristic absorbance in the visible region between 510 and 540 mμ, common to all its metabolites. Alteration of the pH of solutions gives rise to only small changes in wavelength, which usually increases by between 2 and 4 mμ on progressing from acidic to alkaline conditions.

(3) Spectroscopic Determination of Metabolites of Promazine and Chlorpromazine.

Two methods of analysis were employed. The first, using ultra violet spectroscopy, gave a measure of sulphide metabolites in the presence of sulphoxides, and vice versa. The second, using the visible spectrum, gave a measure of the total phenothiazine content of each extract. Both these methods depend on the fact that extinction coefficients are comparable for metabolites and the parent compound. Chemical tests were also occasionally carried out on urine samples to confirm the presence of conjugated fractions (Pg.113)

(a) Determination of Sulphide and Sulphoxide Derivatives (Flanagan's Method.)

(i) Determination of sulphides in the presence of sulphoxides.

The ultra violet spectrum obtained from a mixture of sulphide and sulphoxide derivatives of a drug resembling promazine in spectral characteristics is a combination of the sulphide and sulphoxide spectra. Thus promazine produces five peaks at 230,

252, 272, 300 and 340 mμ and a corresponding spectrum is obtained from the chlorpromazine derivatives. The concentration of sulphide or sulphoxide metabolites in each extract was calculated from this single spectrum by a background cancellation method described by Flanagan et al. (1959).

For determination of sulphide derivatives a straight line was drawn between points A and B on the ultra violet curve corresponding to wavelengths of 236 and 270 mμ respectively. A perpendicular line was then drawn from the major sulphide absorbance maximum (252 for promazine; 255 for chlorpromazine) to intersect AB at C. The difference between the maximum absorbance and the absorbance at C was calculated for each extract. This value was compared with a calibration curve of absorbance against concentration obtained by extracting standard urinary solutions of each drug by Procedure 1 (Pg.101), and calculating the absorbance difference as described.

(ii) Determination of Sulphoxides in the presence of Sulphides.

The sulphoxide content of extracts was also calculated from the ultra violet spectrum by a background cancellation procedure. The absorbance of the peak in the 270 mμ region (272 for promazine; 274 for chlorpromazine), and the absorbance at 315 mμ were measured. The difference in absorbance between these two wavelengths was then compared with a calibration curve of absorbance against concentration. This was prepared by extracting standard urinary solutions of promazine sulphoxide or chlorpromazine sulphoxide by Procedure 1, calculating the absorbance difference

and plotting it against concentration. Using these methods sulphide or sulphoxide concentrations in an extract could be determined down to a concentration of 1 mg/litre. Derivatives detected in lower concentrations than this were classed as trace amounts.

(b) Determination of Phenothiazine Content of Extracts.

The combined sulphide and sulphoxide content of extracts was determined from their visible spectra after reaction with 50% sulphuric acid. The reaction was carried out as previously described, (Pg. 104), and the maximum absorbance in the visible region (515 mμ for promazine; 530 mμ for chlorpromazine) was measured. This value was compared with a calibration graph of absorbance against concentration prepared by extraction of standard urinary solutions of promazine hydrochloride or chlorpromazine hydrochloride by Procedure 1, followed by colour development and absorbance measurement.

Reproducible results were obtained using this method provided manipulations were standardised, and metabolites could be detected in extracts down to a concentration of approximately 2mg/litre. There was also good correlation between amounts calculated by this method, and the amounts obtained by combining the sulphide and sulphoxide contents as determined from the ultra violet spectra. The method finally adopted for quantitative analysis was to calculate the sulphide and sulphoxide contents of each fraction using ultra violet spectrometry, then use the visible spectrum to confirm the findings.

4. Spectroscopic Determination of Metabolites of Acepromazine and Propionylpromazine.

Solutions containing both acepromazine and its sulphoxide, or propionylpromazine and its sulphoxide, did not produce ultra violet spectra with the combined absorbance maxima of the two solutes (c.f. Promazine and chlorpromazine). Instead, they were found to produce one major peak which varied in wavelength between 240 and 255 mμ depending on the proportions of sulphide and sulphoxide present. When the ratio of acepromazine to its sulphoxide was 8:2 it appeared at 244 mμ, and the corresponding wavelengths for ratios of 1:1 and 2:8 were 248 and 253 mμ respectively.

This is probably caused by the proximity in wavelength of the major peak for each form (240 and 255 mμ), causing them to overlap. The other maxima normally found at 280 and 315 mμ for sulphide and sulphoxide respectively were absent, except for cases where the solution was almost entirely composed of one or other derivative.

Since only one major peak is produced by a combination of sulphide and sulphoxide derivatives of such compounds, the separate amounts of sulphide and sulphoxide derivatives in a sample cannot be calculated. Their relative concentrations however, could possibly be roughly calculated from the wavelength of the major peak. Instead the method adopted was to assay samples for total phenothiazine content using colorimetry (Pg.104).

On subsequent examination of spectra of urine extracts it was found that only the glucuronide and sulphate conjugated fractions

gave this single maximum, unconjugated metabolites having a spectrum corresponding to that of promazine sulphoxide (Pg.166). Thus unconjugated metabolites were assayed by ultra violet spectroscopy as for promazine sulphoxide and the conjugated fractions were analysed by colorimetry.

(5) Qualitative Tests for Conjugated Metabolites.

Before quantitative analysis of conjugated metabolites their presence was usually first ascertained using simple qualitative tests.

(a) Glucuronide Conjugated Metabolites.

5 mg naphthoresorcinol were dissolved in 0.5 ml concentrated hydrochloric acid and an equal volume of urine was added. The mixture was boiled for 1 minute, cooled, and extracted with an equal volume of ether. The presence of glucuronides produced a purple colouration in the organic layer. Control urine was found to produce only a very faint purple colouration using this reaction.

(b) Sulphate Conjugated Metabolites.

5 mg barium chloride were dissolved in 0.5 ml 2N hydrochloric acid and an equal volume of urine was added. The solution was centrifuged and the supernatant liquid was boiled for 1 minute. On cooling any turbidity or precipitation showed the presence of sulphates.

E. Thin Layer Chromatography.

(1) Extraction Procedures.

Procedures were exactly the same as for quantitative analysis,

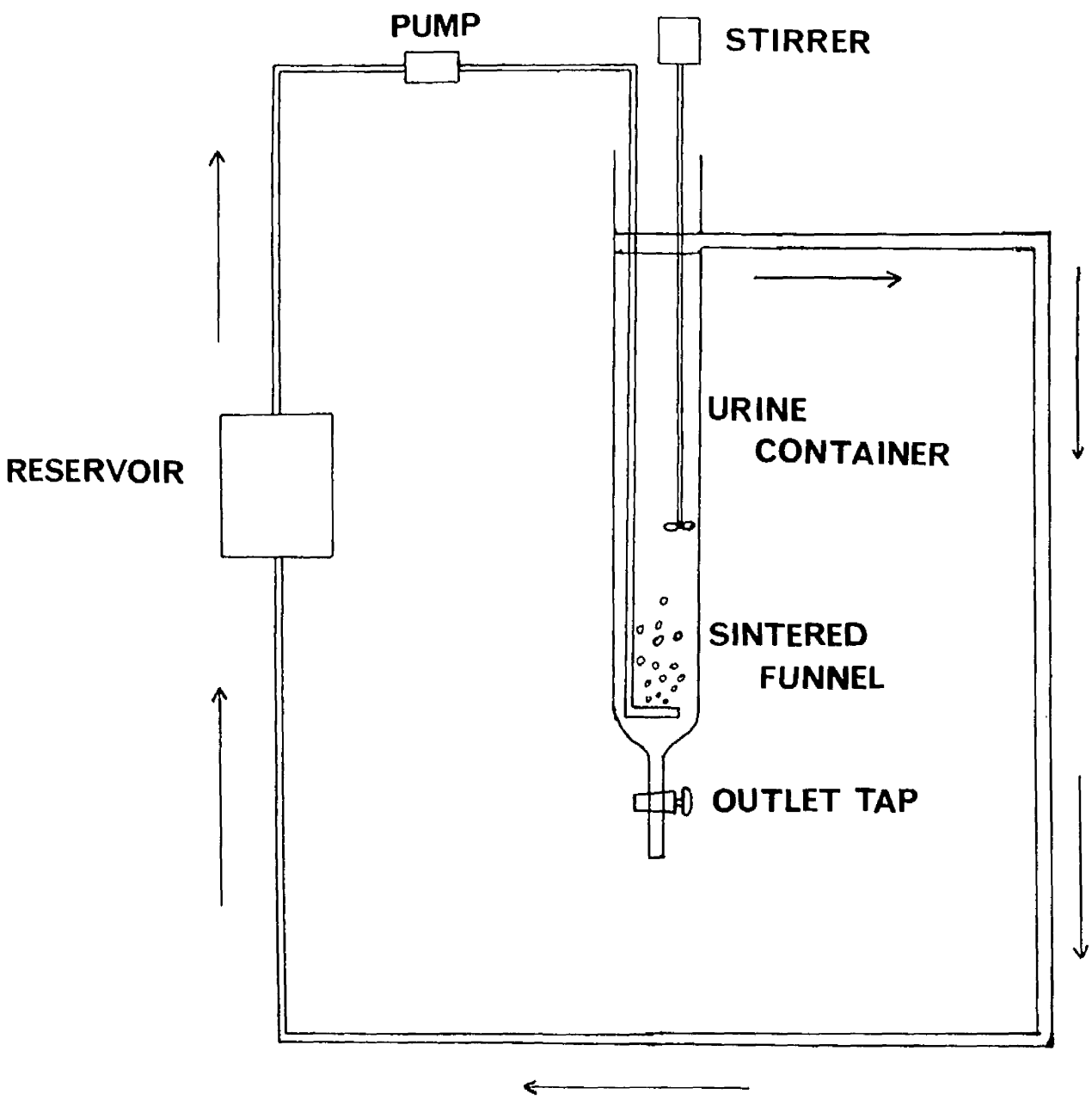
using proportionate volumes of solvent, the final stage being omitted in each case. To obtain a qualitative picture of excretion using thin layer chromatography (Pg.198), 300 ml of urine wereextracted, whereas for structural determination purposes, and occasionally for gas liquid chromatography, much larger volumes were continuously extracted. After washing the extract as described, (Pg.102), instead of taking the metabolites into hydrochloric acid, it was dried over anhydrous ammonium sulphate, evaporated to dryness under reduced pressure, and redissolved in a minimum amount of ethylene dichloride or methanol.

(2) Continuous extraction.

The apparatus employed is shown in Figure 13, and is designed to allow for continuous extraction by any solvent. The glass tube and sintered glass funnel are not attached to the urine container, so that the depth of the funnel in the urine can be altered. For solvents denser than urine the funnel is positioned just below its surface. The principle is that solvent from the reservoir (an aspirator) is pumped through the glass tube by means of a peristaltic pump to the sintered glass funnel, where it is dispersed into the urine as small globules. Dispersion of solvent is further aided by the mechanical stirrer, which causes the globules to spiral downwards through the urine. The solvent collecting at the base of the container is returned through the open tap to the reservoir, under the action of the pump, and is subsequently recycled.

For solvents less dense than urine the situation is as shown in the diagram, the sintered glass funnel being positioned at the

FIGURE 13



CONTINUOUS EXTRACTION APPARATUS

base of the container, and the outlet tap is closed. In this case, the solvent globules spiral upwards to the head of the urine column just below the outlet tube, and the collecting solvent is drawn back to the reservoir by the action of the pump. Both methods have been used for extraction, over 24 hours, of volumes of urine up to three litres.

(3) Preparation of Plates.

Chromatography was carried out on 20 x 20 cm glass plates coated with a 250μ layer of MN Kieselgel (500μ for preparative work). The plates were activated by heating for 30 minutes at 100^{o}C, and stored in a desiccator until required. 2-10 aliquots of extracts were spotted and the plates were developed to a height of 15 cm. In order to obtain a qualitative picture of metabolism over prolonged periods (Pg. 198) layers were divided into 1 cm strips and 10λ aliquots of extracts from successive 8 hour samples were spotted on consecutive strips.

(4) Solvent Systems.

Each metabolic fraction from promazine or chlorpromazine was run in three solvent systems. For the unconjugated fraction the systems used were:

1 Methanol - Acetic Acid - Water (5:3:2)

11 Ethanol - Acetic Acid (1:1)

111 Chloroform - Acetone - Diethylamine (2:7:1) (Goldenberg et al., 1965).

For both conjugated fractions the systems were:

1 Acetone - isopropanol - 1% ammonia (9:7:4) (Goldenberg

et al., 1965).

II Ethyl Acetate - Methanol - Diethylamine (7:2:1)

III Methanol - Acetic Acid - Water (5:3:2)

In the case of acepromazine and propionylpromazine only two solvent systems were used since so few reference compounds were available. Unconjugated extracts were developed in the methanol-acetic acid-water system and the conjugated fraction in the system acetone-isopropanol-1% ammonia (8:7:5).

All diagrams of chromatograms shown in Chapter VI unless otherwise stated, were recorded after running in methanol-acetic acid-water (5:3:2) for unconjugated metabolites, and in acetone-isopropanol-1% ammonia (9:7:4) for the conjugated fractions.

(5) Location of Metabolites.

(I) Due to their fluorescent properties, metabolites were first visualised under ultra violet light (254 mμ). This method of location was also used when elution of spots was required, as the short periods of irradiation did not appear to alter their molecular structures.

(II) Metabolites were also located by spraying with 50% $^{v}/_{v}$ sulphuric acid as described by Salzman and Brodie (1956). This is a general reagent for phenothiazine derivatives detecting both sulphides and sulphoxides.

(III) Ferric chloride was used for location of sulphide derivatives. The spray consisted of a 1% $^{w}/_{v}$ solution of ferric chloride ($FeCl_3.6H_2O$) in 2.5 N HNO_3.

(IV) In addition to the sulphuric acid spray, periodate

was used for the detection of hydroxylated metabolites (1% aqueous sodium metaperiodate).

(V) Ninhydrin was used for location of didemethylated derivatives. The spray consisted of a 1% $^{w}/_{v}$ solution of ninhydrin in 10% $^{v/v}$ aqueous acetic acid. After spraying, the plate was heated at 100^{o}C for 10 minutes.

(VI) Nitroprusside was used to identify monodemethylated derivatives. The reagent consisted of two solutions, A and B, which were mixed in equal proportions immediately before use. A was a 3% solution of sodium nitroprusside in 10% aqueous acetaldehyde, and B was a 2% aqueous solution of sodium carbonate.

All spray reagents were used on a single plate. This was achieved by dividing the layer into 1 cm strips as previously described and spotting equal aliquots (10 λ) of the extract on each strip. After development, three consecutive strips were sprayed with each reagent, two uncoated glass plates being used to shield the layer on either side. When a spray produced no coloration, further plates were spotted with progressively larger aliquots of extract (up to 50 λ) to confirm that no reaction had taken place.

(6) Elution of metabolites.

For elution purposes, 0.5 ml aliquots of extract were streaked on 20 x 20 cm layers 0.5 mm in thickness. The aliquots were applied using a pasteur pipette, with a plug of cotton wool fitted at the tip, to avoid damage to the layer and control the flow of extract. After development the streaks were either visualised under ultra violet light, or located by spraying the

end strips of the plate with 50% sulphuric acid, the middle portion being protected by a glass plate. In both cases the position of streaks were outlined using a metal marker.

Each area of silica containing a phenothiazine derivative was then scraped off, and corresponding areas from different plates were combined in conical flasks. The minimum amount of solvent necessary for extraction (5-10 ml) was added to each flask, and the mixtures placed for 1 hour on an automatic shaker. After shaking, the mixture was centrifuged and the supernatant eluate decante

Individual metabolites were eluted for ultra violet spectroscopy using 0.1N hydrochloric acid. Using reference compounds it was found that the minimum amounts of metabolites which would give interpretable ultra violet spectra on elution into 5 ml of acid ranged between 5 and 20 µg. Sulphide derivatives of chlorpromazine could be detected down to 5 µg, whereas both hydroxylated and sulphoxide derivatives were not detectable below 8 µg. The corresponding figures for promazine were 8 µg and 12 µg. The minimum amount of acepromazine and its sulphoxide were 10 and 20 µg respectively. Attempted elution with organic solvents proved unsuccessful, although small quantities were occasionally eluted by methanol. The difficulties are considered in the discussion (Pg. 262).

F. Gas Liquid Chromatography.

(1) Packing of Columns.

Appropriate amounts of SE-30 (1% or 5%) and Chromosorb W were weighed out and the SE-30 dissolved in toluene. When column coating was required Epicote 1001 was also added to the toluene in 10% concentration. The Chromosorb was then added to this solution and the toluene allowed to evaporate. The packing was drawn into the chromatographic column by suction from a vacuum pump, the column being constantly shaken to ensure uniformity of packing. Each column was preconditioned by heating for 24 hours at a temperature 50°C above the normal operating temperature.

(2) Operating Conditions.

The optimum operating temperature for the stainless steel columns packed with 5% SE-30 was 250°C. The conditions were: Column - 5 x $\frac{1}{8}$" o.d. stainless steel; Stationary Phase - 5% SE-30; Support - Chromosorb W (50-80 mesh); Column Temperature 250°C; Injector Temperature 275°C; Detector Temperature 275°C; Carrier Gas Flow Rate - 40 ml/minute. Such columns were also used over a range of temperatures between 150 and 300°C, and in each case injector and detector temperatures were 25C° above that of the column.

The optimum operating temperature for the glass columns packed with 1% SE-30 was 200°C. The conditions were: Column- 5' x $\frac{1}{8}$" o.d., glass; Stationary phase - 1% SE-30; Support-Chromosorb W (50-80 mesh); Column Temperature 200°C; Injector Temperature 225°C; Detector Temperature 225°C; Carrier Gas Flow Rate -

30 ml/minute.

The L.K.B. gas chromatograph-mass spectrometer was also fitted with glass columns (8' x $\frac{1}{4}$") packed with 1% SE-30. The operating temperatures ranged from 150 - 300°C.

G. Miscellaneous Procedures.

(1) Oxidation of Propionylpromazine to Propionylpromazine Sulphoxide.

Since samples of propionylpromazine sulphoxide were unavailable this compound was synthesised from the commercially available sulphide in order to determine its spectral and chromatographic properties. The method employed was based on that of Kano and Fujimoto (1957) for oxidation of chlorpromazine to its sulphoxide.

50 ml propionylpromazine phosphate injectable solution, (Combelen - 10 mg/ml), was adjusted to pH 9.5 using N ammonia solution and extracted with 3 x 10 ml ethylene dichloride. The extract was then evaporated to dryness under reduced pressure and the residue taken up in 5 ml ethanol. 3 drops of 30% hydrogen peroxide were added and the solution stirred.

This solution was then refluxed for 4 hours, and the solvent was evaporated off. The residue was recrystallised three times from acetone and subsequently used for ultra violet spectroscopy and thin layer chromatography. The yield of sulphoxide was less than 10%.

Attempts at oxidation of some of the standard reference compounds (e.g. demethylated and hydroxylated derivatives) were unsuccessful.

This was probably due to the small amounts of such compounds available, and the low yield obtained from the reaction.

(2) Determination of β-glucuronidase activity in horse urine.

Two samples of urine, a test and a control, (1ml), were centrifuged, the urine decanted, and adjusted to pH 4.5 using 1.1 ml acetate buffer.* 0.1 ml 0.01M phenolphthalein-glucuronic acid substrate was added to the test sample and both were incubated at 37°C for 24 hours. 1 ml 10% aqueous sodium carbonate was then added to both samples, followed by 1 ml substrate to the control. Each was made up of 10 ml with distilled water and the absorbance of the test at 550mμ read against the control.

The glucuronidase activity was read from a calibration graph of absorbance v activity. This was prepared by adding different volumes of phenolphthalein (0.05, 0.1 & 0.2 ml) to 1 ml urine and 1 ml sodium carbonate, making up to 10 ml with water, and reading the absorbance against a urine blank. One unit of activity was defined as the amount of enzyme liberating 1 μg phenolphthalein under the conditions of the experiment.

* 3.25ml acetic acid and 5.79 gm sodium acetate were made up to 450 ml water, the pH was adjusted to 4.5 and the volume made up to 500 ml.

STORAGE AND HANDLING OF HORSE URINE.

V STORAGE AND HANDLING OF HORSE URINE.

A. Precipitate from Alkaline Urine.

As previously described (Pg.103) a white precipitate is obtained on rendering horse urine alkaline. The compound was isolated after centrifuging, washed with water, and dried in a desiccator. The resulting white powder did not burn and was thus presumed to be inorganic. It was insoluble in organic solvents, water, 0.1N ammonia, and 0.1N hydrochloric acid.

Infra red spectroscopy showed very strong peaks at 1450 and 880cm^{-1} which suggested that a carbonate was present. This was confirmed by production of carbon dioxide on reaction with chromic acid. Group separation and a subsequent flame test showed the presence of calcium. This was also confirmed by a white precipitate with ammonium oxalate.

Microscopic examination of the precipitate showed mostly the crystal forms of calcium carbonate, amorphous calcium carbonate, and calcium phosphate although traces of oxalate crystals were also present. Biochemical analysis of electrolytes before and after centrifuging also showed a large decrease in calcium levels (180mg/100ml to 6 mg/100ml). Thus the major constituent of the precipitate was calcium carbonate.

B. Interfering compounds in urine.

(1) Introduction.

During preliminary quantitative experiments it was noted

that an additional peak appeared on the ultra violet spectra of some extracts of urine stored at 4°C. This peak at times appeared within 24 hours of sample collection, and always appeared within one week, thereafter increasing in intensity with length of storage of the urine. Since the maximum occurred at 278 mμ it tended to interfere with the ultra violet determination of metabolites by masking the phenothiazine peaks. Figure 14 shows a typical spectrum of this contaminant superimposed on the spectrum of promazine sulphoxide.

Due to the gradual increase in intensity of this peak with time it was postulated either that some organism, dormant in the urine, was slowly producing a compound responsible for the absorbance, or that microbial organisms from the air, or sample containers, were acting on a substrate in the urine giving rise to the contaminant. On the other hand it could be caused by slow reaction between endogenous compounds in the urine. The theory of bacterial action was supported by the fact that incubation of control urine at 37°C for 24 hours, immediately after collection, always produced a very intense absorbance at 278 mμ.

It was also noted that there is an increase in β-glucuronidase activity with time in urine stored at 4°C. Figure 15 shows a typical increase in such activity in a control urine sample, and increase in the absorbance of its extract, over a period of 1 month. For the first 15 days the values follow each other closely, after which the glucuronidase activity falls off rapidly, the absorbance

FIGURE 14.

SPECTRA OF PROMAZINE SULPHOXIDE AND THE INTERFERING COMPOUND.

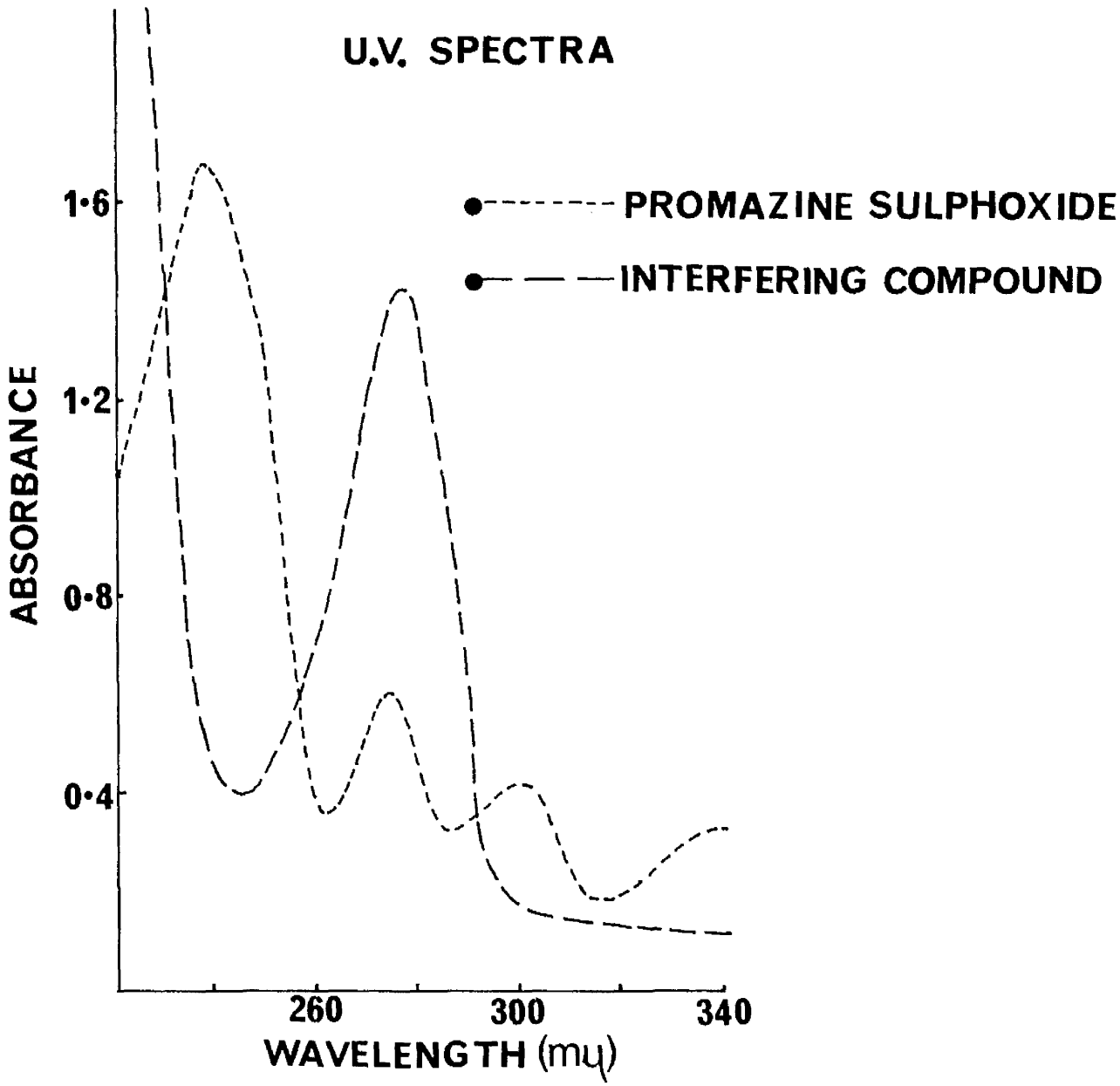
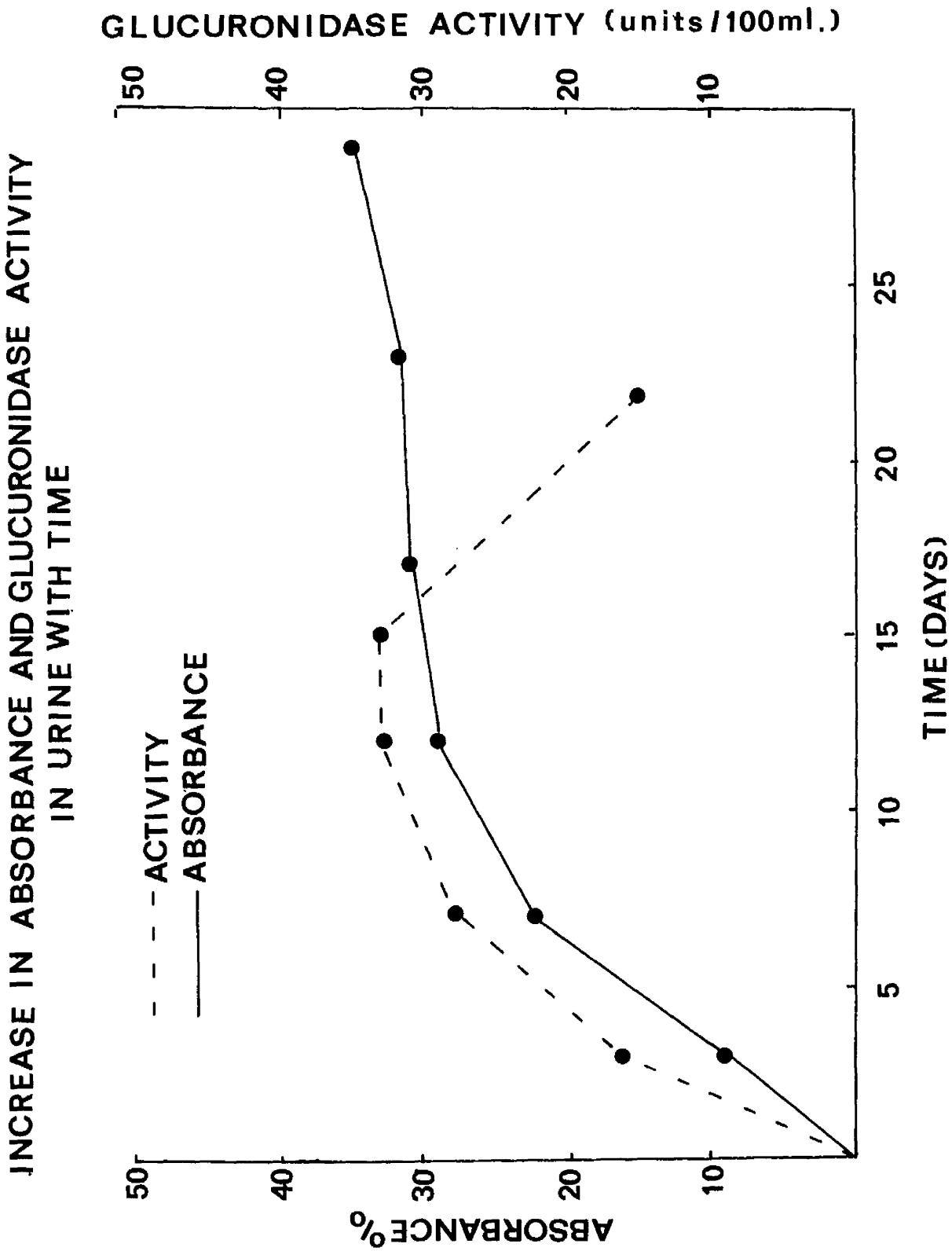


FIGURE 15.


INCREASE IN ABSORBANCE AND GLUCURONIDASE ACTIVITY IN URINE WITH TIME
GLUCURONIDASE ACTIVITY (units/100ml.)
ABSORBANCE %
TIME (DAYS)
ACTIVITY
ABSORBANCE
50
40
30
20
10
5
10
15
20
25

remaining at a fairly constant level. It has also been found after incubation of control urines at 37°C that β-glucuronidase levels usually exceed 40 units/100 ml., having been negligible before incubation.

It was at first thought that this build up in activity resulted in the hydrolysis of endogenous unidentified glucuronide conjugates, giving rise to a gradually increasing interference on spectra of extracts. After two to three weeks this interference reached a maximum either due to the glucuronide substrate being exhausted, to the fall off in activity, or to a combination of both. However, if such substrates are present they would be hydrolys during the normal β-glucuronidase incubation after sterilisation (Pg.104). Thus the rise in activity seems to be a coincidence. The process, however, does reach a point of exhaustion. This can be seen from the fact that control samples stored at 37°C, extracted by procedure 1, then subsequently incubated with β-glucuronidase, show very little interfering absorbance (Pg.133).

(2) Treatment of Urine.

Since prolonged incubation procedures are involved in the assay of conjugated metabolites it was attempted either to remove the interfering compound when formed, to eliminate the organism by sterilisation, or to inhibit its action by more sophisticated methods of storage.

(a) Effects of Ion Exchange Resins.

Initially, attempts were made at removing the contaminant. Eiduson and Wallace (1958), whilst determining phenothiazine

derivatives in human urine, removed 95% of the phenothiazine content from standard samples using a cation exchange resin, Amberlite IRC50. An anion exchange resin, Dowex AG3X4, was used by Forrest et al. (1960) to remove indican and various contaminants from human urine.

This suggested the possibility of using one of these resins to selectively remove either the drug and its metabolites, or the interfering compound, from samples of horse urine. A series of preliminary experiments was carried out to evaluate their usefulness.

Standard solutions of promazine hydrochloride (~6mg/l) and promazine sulphoxide (~12mg/l) were made up in a series of phosphate buffers (pH 6.0, 7.1 and 8.3), and a borate buffer (pH 9.3). The ultra violet spectrum of each solution was then recorded.

1.5 gm. Amberlite IRC50 resin was added to 50 ml of each standard promazine solution, and the mixture was shaken for 4 hours. After filtering, the ultra violet spectrum of each sample was run using the appropriate buffer as reference. The loss in phenothiazine content was then measured from the percentage decrease in absorbance of the major peak. The same procedure was carried out with 50 ml samples of the standard promazine sulphoxide solutions. A similar procedure was also adopted using Dowex resin, 1 gm of this compound being added to each of the standard solutions.

The experiment wasalso carried out on a sample of control urine which showed the interfering absorbance on the ultra violet

pH	DOWEX			AMBERLITE		
	PROMAZINE	SULPHOXIDE	IMPURITY	PROMAZINE	SULPHOXIDE	IMPURITY
6	●●●●	●●●●	●●●	●●●●	●●●●	●●
7·1	●●●●	●●●	●●●	●●●	●●	●●
8·3	●●●●	●●●	●●●	●●	●	●●
9·3	●●●●	●●	●●●	●●	●	●●

TABLE 3.

●= 0–25 % REMOVED

●●=25–50 % REMOVED

●●●= 50–75 % REMOVED

●●●●= 75–100 % REMOVED

PERCENTAGE REMOVAL OF PROMAZINE, PROMAZINE SULPHOXIDE, AND INTERFERING COMPOUNDS BY DOWEX AG3×4 AND AMBERLITE IRC50 RESINS

spectra of its extracts. It was divided into four portions which were then adjusted to pH6, 7.1, 8.3 and 9.3 respectively. 1.5 gm amberlite IRC50 resin was added to a 50 ml aliquot of each portion. The mixture was shaken for four hours, filtered, extracted by Procedure 1 (Pg.101), and the ultra violet spectrum recorded. The same process was repeated for each of the four portions using 1 gm Dowex resin and any loss in absorbance was recorded.

The results of the experiment (Table 3), show that over the pH range examined, although the Dowex resin is very efficient in removing the interfering compound, it is even more efficient at removing promazine and promazine sulphoxide. The Amberlite resin, especially at alkaline pH, does not remove a great amount of promazine or promazine sulphoxide, but at the same time, is not very effective in removing the interfering compound.

Since these initial experiments did not produce the desired results, and since it seemed much time consuming work would be involved in finding a selective resin, it was decided to investigate sterilisation procedures.

(b) Effects of Filtration.

The possibility of sterilising the urine using a Seitz filter was investigated. A sample of control urine was extracted by Procedure 1, the ultra violet spectrum of the extract was recorded, and no interfering peaks were found. The remaining urine was divided into 2 portions. One half was filtered through a 14 cm HB/EKS Carlson-Ford bacterial filter pad, then stored under sterile conditions at $4^{o}C$ in a refrigerator. The other portion was put

straight into storage. An aliquot of each was submitted for bacterial examination by attempting culture on Blood Agar. The filtered sample proved to be completely sterile, whereas the unfiltered one showed most of the common microbes found in any contaminated medium.

Samples of each portion were extracted by Procedure 1 and 2 at random intervals, over a period of six weeks, and ultra violet spectra were recorded. The sterilised fraction showed no abnormal absorbance over this period using either procedure. The other fraction gave an abnormal peak when treated by Procedure 1 which gradually increased in intensity throughout the experiment. When treated by Procedure 2 this portion produced a very intense absorbance in every sample. These results suggested that Seitz filtration might be of use in preventing interference, due to contamination, on the ultra violet spectra. However, the possibility that the drug and its metabolites might be removed by the filter had also to be investigated.

Promazine hydrochloride was added to a control sample of urine to give a concentration of $\sim$ 6mg/l, and the sample was divided into two portions. One portion was filtered through the Seitz filter and the other was used as a control. Both fractions were extracted by Procedure 1 and their ultra violet spectra recorded. On comparing the absorbance of each portion it was found that approximately 80% of the promazine had been removed by the Seitz filter. On repeating the experiment using promazine

sulphoxide (~12mg/l), approximately the same amount was removed. This ruled out the possibility of using this method of sterilisation to remove the contaminant.

A duplicate set of experiments was also carried out using a millipore filter instead of the Seitz filter. Again no signs of interfering absorbance were noted in extracts over 6 weeks from the filtered urine, but approximately 50% of the added promazine or promazine sulphoxide was removed.

(c) Effects of storage.

Various methods of storage were also investigated in an attempt to inhibit formation of the contaminant. A pooled sample of control urine was divided into 100ml aliquots in sterile bottles. Groups of six samples were then treated and stored as described. Each group was stored in the dark to avoid the possibility of photochemical reactions.

Group I. Samples were stored at $4^{o}C$ in stoppered bottles.

Group II. Nitrogen was bubbled through the urine for 15 minutes, the bottles were then stoppered and stored at $4^{o}C$.

Group III. Samples were adjusted to pH1.1, (the pH of 0.1NHCl), using concentrated HCl. The bottles were then stoppered and stored at $4^{o}C$.

Group IV. Samples were stored in an incubator at $37^{o}C$ in stoppered bottles.

Group V. Samples were stored in sealed polythene bags at $-20^{o}C$ in a deep freeze.

Group VI. Samples were stored under a layer of toluene at 4°C in stoppered bottles.

GroupVII. Samples were heated in a boiling water bath for five minutes, cooled and stored at 4°C in stoppered bottles.

Immediately after collection a 100ml sample of the pooled urine was assayed by Procedure 1 to ensure no interfering compound had been originally present. One sample from each treated group was then assayed at weekly intervals by Procedures 1 and 2.

Results are shown in Table 4. After analysis by Procedure 1, the concentration of the interfering compound gradually increased in four of the groups,i.e. that stored at 4°C, that stored under toluene, that stored under nitrogen, and that stored at pH 1.1. In addition, the group stored at 37°C, produced a very intense absorbance in every case. Thus these five methods of storage would not eliminate the formation of the interfering compound and were not further considered. On the other hand, by the end of three weeks, neither the group stored at -20°C, nor the heated group,produced any absorbance when assayed by Procedure 1. Thus it was decided to adopt one of these methods of storage.

On considering samples assayed by Procedure 2, it was found that every sample, with the exception of the group stored at 37°C, gave an intense peak at 278mμ showing that incubation accelerates production of contaminants. Also since those samples which had been stored at 37°C, after extraction by Procedure 1 gave very weak absorbance on further incubation, it was assumed

TABLE 4.

	4^0C	TOLUENE	N_2	ACID	37^0C	-20^0C	HEATING	WEEK
UNCONJ.	2	2	2	2	6	1	1	FIRST
	4	4	3	2	6	1	1	SECOND
	4	4	3	3	6	1	1	THIRD
CONJ.	5	5	5	4	3	4	4	FIRST
	5	5	5	5	2	6	5	SECOND
	5	5	5	5	2	6	5	THIRD

$\%_0$ ABSORBANCE	
0–10	1
10–30	2
30–50	3
50–70	4
70–100	5
SAMPLE DILUTED	6

ABSORBANCE OF INTERFERING COMPOUNDS FROM URINE UNDER DIFFERENT FORMS OF STORAGE.

that the source of the interfering compound had been exhausted during the initial storage period. However, the peaks were not so weak as to render this method of storage applicable.

(d) Sterilisation for Procedure 2.

Although samples which had been treated either by heating or by deep freezing could now be assayed by Procedure 1 without interference, the incubation stage for conjugated metabolites still gave rise to interfering absorbance. Thus further experiments were carried out in an attempt to eliminate contaminants in assays by Procedure 2. This involved more stringent sterilisation of the urine, and afterwards carrying out the procedure under completely sterile conditions.

A 100 ml aliquot of control urine was assayed by Procedure 1 and was found to have no abnormal absorbance. The residual urine was then extracted by Procedure 2, and produced a peak at 278 mμ so confirming the presence of organisms capable of producing the contaminants. Three 100 ml aliquots of the same urine were then analysed under sterile conditions by Procedure 2. The first sample was extracted without any previous treatment, the second was first heated for 15 minutes at 100 °C, and the third was first heated for 10 minutes at 100°C at three successive 24 hour intervals. The first sample showed interfering absorbance at 278 mμ, and the second still showed the presence of the impurity, although the intensity of the absorbance had halved. In the third sample only a trace of the interfering compound was found. Thus it seemed that the second, more rigorous method of sterilisation would

prevent the formation of contaminants during the prolonged incubation step.

To ascertain the applicability of this technique to the assay of urine samples containing phenothiazine metabolites, standard urinary solutions of promazine (6mg/l) and promazine sulphoxide (12 mg/l) were made up. Two 100 ml aliquots of each solution were extracted by Procedure 1 firstly without treatment, and secondly after heating for 10 minutes at 3 successive 24 hour intervals. The resulting spectra showed that the heating had no noticeable effect on the concentration of either promazine or its sulphoxide.

It was thus decided to store samples at -20^{o}C when any length of time would be involved between collection and analysis. Also when analysis of conjugated metabolites was required the residual urine from Procedure 1 was first sterilised repeatedly as described.

LEGEND FOR DIAGRAMS

Unless otherwise stated the symbols employed in graphs and diagrams are as follows:

(a) Quantitative Studies

- ● = Total metabolites
- □ = Unconjugated sulphoxides
- △ = Glucuronide sulphoxides
- ○ = Glucuronide sulphides
- ■ = Sulphate conjugated sulphides

(b) Thin layer chromatography

(i) Solvent systems.

Unconjugated metabolites:- Methanol-acetic acid-water (5:3:2)

Conjugated metabolites:- Acetone - isopropanol - 1% ammonia (9:7:4)

(ii) Spray reagent - 50% sulphuric acid

(iii) Colour formation


Purple


Reddish pink


Bluish purple


Yellowish brown

VI RESULTS

A. Effects of Drugs.

After administration of each drug, any visible effects on the animal were noted. With the larger doses used for quantitative studies (Pg. 99) the usual signs already described after tranquilliser administration (Carey and Sanford, 1963) were again noted for every drug. Such effects included lowering of the head, drooping of eye/lids and lower lip, resting the hind leg, yawning, snoring, slight unsteadiness when standing, and a general dopey appearance. In geldings the penis was relaxed.

After intramuscular administration, reaction to the drug was usually first noted between 20 and 40 minutes after injection, the drug having maximum effect over the first 4 to 8 hours. However effects were still noticeable after 12 hours, and occasionally up to 24 hours. After oral administration, usually one hour elapsed before the drug showed a noticeable effect, which was most marked for a further 3 to 6 hours. Signs of tranquillisation had usually disappeared by 18 hours after administration.

Every drug caused a lowering in rectal temperature, usually of the order of 1 - 2F^{o} over the first 4 hours after administration. However, on one occasion, (Horse 2 - Promazine I.M. - 5 mg/Kg), a drop of 4F^{o} was recorded within 3 hours. The temperature had usually returned to normal by 12 hours after administration.

Administration of each drug also usually gave rise to a slight tachycardia, the increase in pulse rate being approximately

5 - 10 beats/minute. However, on one occasion, (Horse 2 - Promazine I.M. - 5 mg/Kg.), an increase of 28 beats/minute was recorded at 6 hours after injection. In every case the rate had returned to normal by 12 hours. Effects on respiration were variable and unpredictable. In some cases no difference was noted, whereas on other occasions small rises or falls were recorded, unrelated to the drug or route of administration.

The excitement reaction already described did not occur, but untoward reactions were noted on two occasions. On the first occasion, after intramuscular administration of chlorpromazine (2mg/Kg) to horse 4, the animal appeared to react normally to the drug. Thus, after 4 hours, a bar, which had been placed across the top half of the loose box door in case of abnormal reaction, was removed. Within 2 hours the animal tried to leap the door but was caught by the hind legs in the process causing considerable cuts and grazing. On the only occasion that propionylpromazine was administered (Intramuscular - 0.5 mg/Kg), the horse (7) at first showed slight signs of tranquillisation. However, at 15 minutes after injection it rapidly became very dopey, unsteady on its feet and pawed the ground, and within 3 minutes went down anaesthetised. Four hours elapsed before the horse returned unsteadily to its feet, and it still appeared rather groggy after a further 12 hours. The period of anaesthesia was accompanied by a fall in rectal temperature of $5F^{o}$.

After administration of the smaller doses of each drug used for limit of detection studies (Pgs.224,230 236.), visible signs were

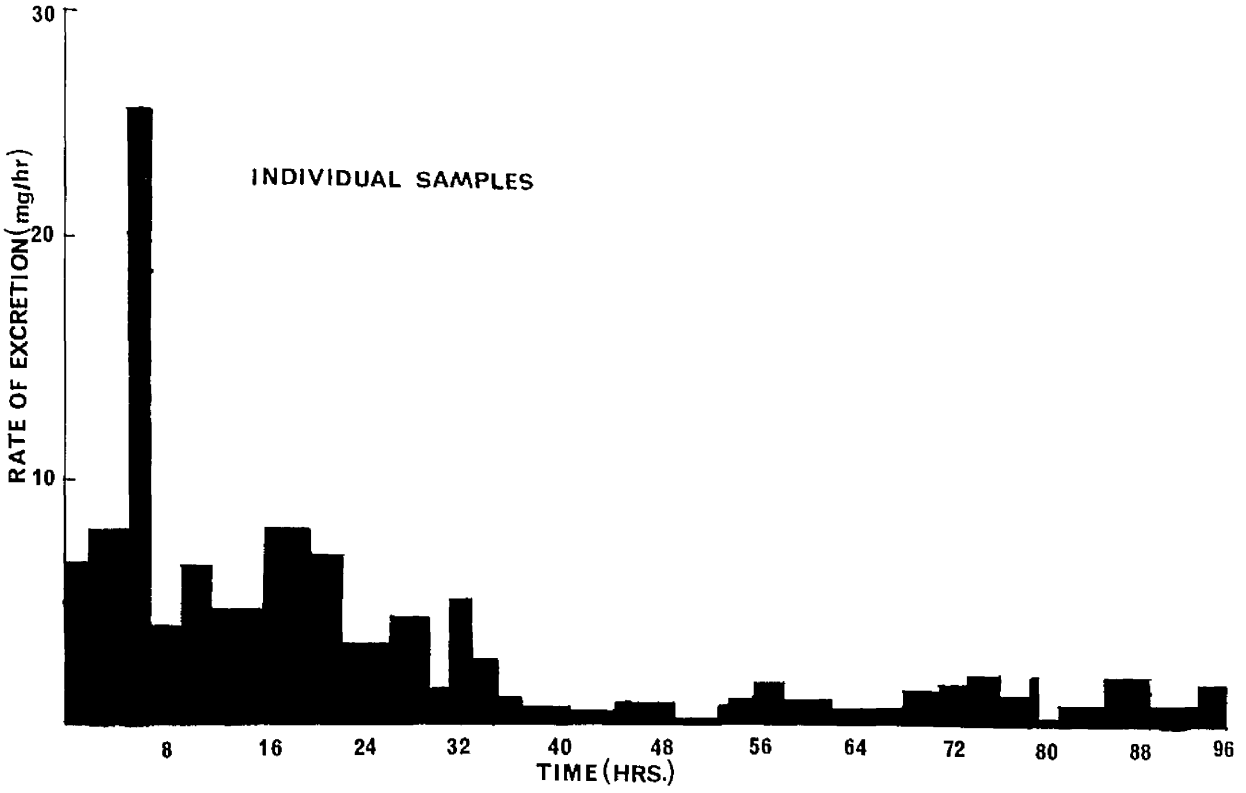
only noted after the largest of the three doses and only to a slight extent.

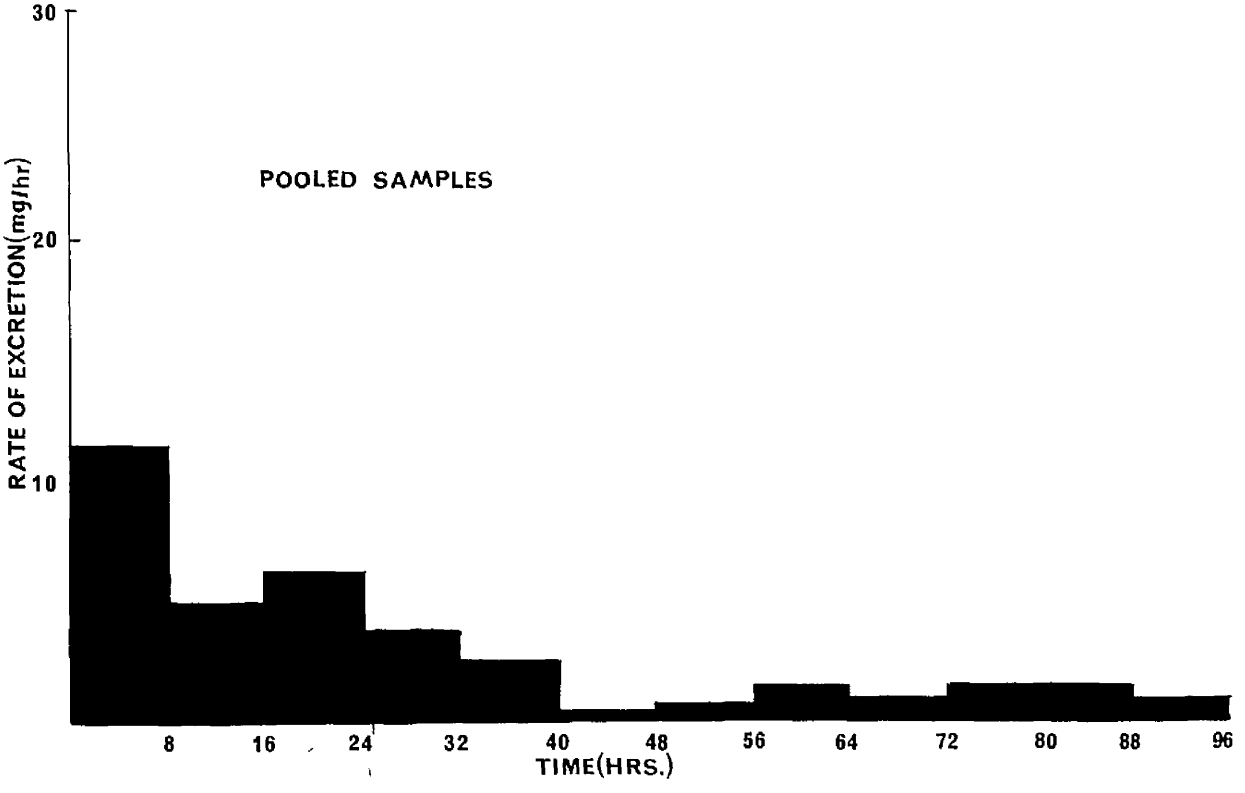
8. Quantitative studies of metabolism.

Initially quantitative studies were carried out on each urine sample passed. However, results obtained using this method were difficult to analyse due to large variations in concentration over short periods of time, and the difference in time intervals between successive samples. In addition, investigation of every urine sample meant analysis of a large number of samples, which was later found unnecessary. Typical graphs obtained using this procedure and the method subsequently adopted are shown in Figure 16. It can be seen in the former case that the interval between samples differs considerably, and large differences occur in the amounts excreted from sample to sample, making the results very different to interpret. In the latter case, with larger regular intervals between samples, the variations in the amounts excreted in successive samples are not so pronounced.

The method adopted was to pool samples obtained over fixed consecutive periods of time, analysis being carried out on an aliquot from each pooled sample as previously described (Pg.101) Periods of 8 hours were chosen since this seemed to give the most uniform results. The concentration of total metabolites, and their concentration in individual metabolic fractions, was determined for each pooled sample. The amount excreted over successive 8

FIGURE 16.


INDIVIDUAL SAMPLES
RATE OF EXCRETION(mg/hr)
30
20
10
8
16
24
32
40
48
56
64
72
80
88
96
TIME (HRS.)


POOLED SAMPLES
RATE OF EXCRETION(mg/hr)
30
20
10
8
16
24
32
40
48
56
64
72
80
88
96
TIME(HRS.)

hour intervals was then calculated by multiplying this concentration by the urinary volume for the period, and the rate of excretion (mg/hr) was obtained by dividing each amount by 8. This rate was plotted against time for successive pooled samples to give an excretion pattern.

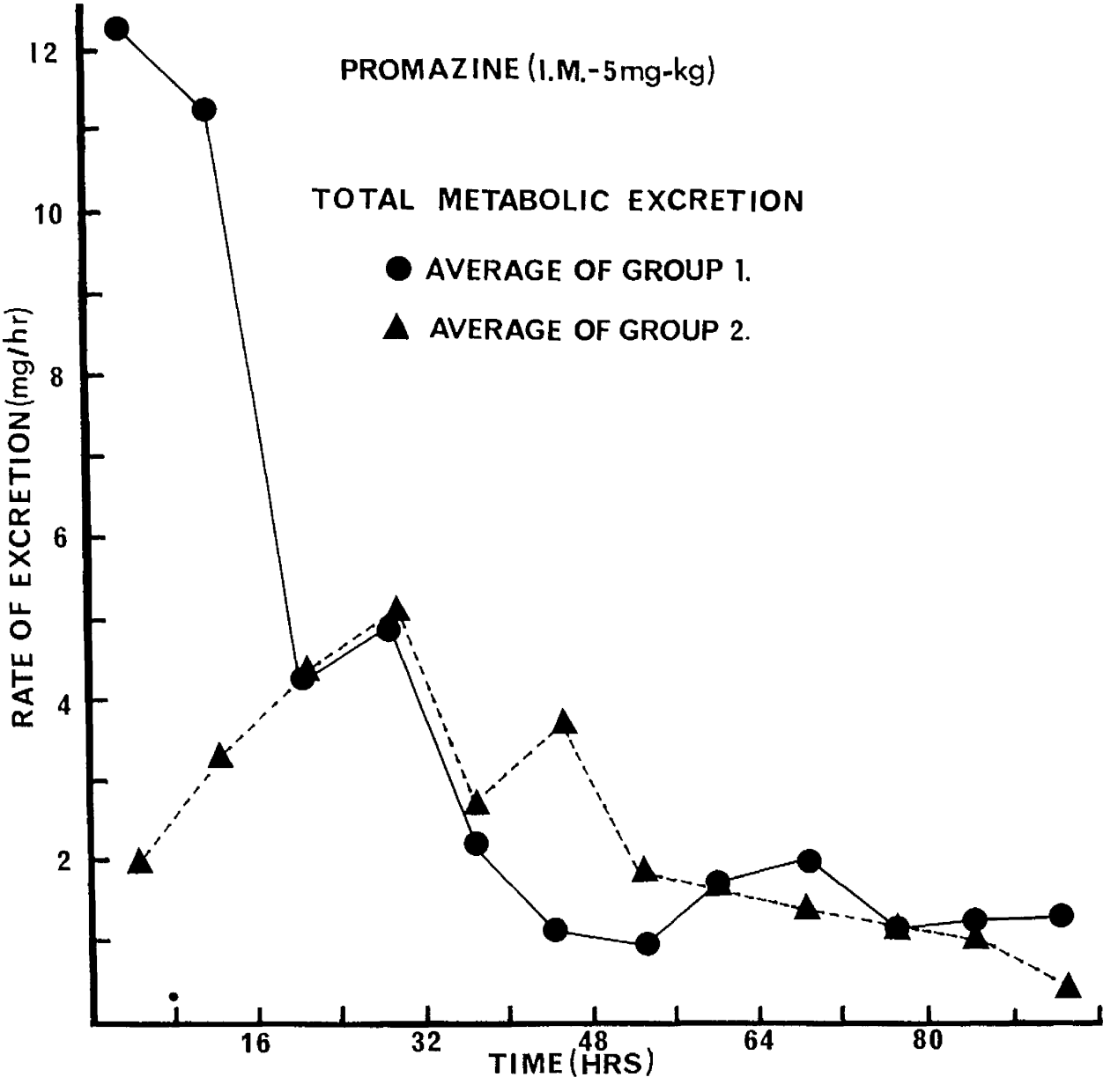
Such graphs were plotted for each drug. The doses and methods of administration employed are described on page 99 . Metabolites were analysed as previously described in six groups dependent on their chemical structure, i.e. sulphoxide derivatives, (all metabolites having a sulphoxide grouping), and sulphide derivatives, (all metabolites in which the ring sulphur atom is unoxidised, including N-oxides), of the unconjugated, glucuronide conjugated and sulphate conjugated fractions. Rates of excretion were plotted for each group, and combined to give the total rate of excretion.

(1) Promazine.

(a) Intramuscular Administration.

Rates of excretion were plotted for six experiments (Horses 1, 2, 4, 7, 5a & 5b) after intramuscular administration of promazine hydrochloride (5mg/Kg as a 5% solution). It was noted that the patterns for the rate of excretion of total metabolites fell into two groups. A graph depicting the average of these rates for each group is shown in Figure 17. In the first group, noted in three experiments, excretion rapidly reached a maximum ($\sim$ 12mg/hr) within 8 hours of injection, then tailed off gradually and irregularly, whereas in the second group the maximum ($\sim$ 5mg/hr) was not attained

FIGURE 17.


PROMAZINE (I.M.-5mg-kg)
TOTAL METABOLIC EXCRETION
AVERAGE OF GROUP 1.
AVERAGE OF GROUP 2.
RATE OF EXCRETION (mg/hr)
12
10
8
6
4
2
16
32
48
64
80
TIME (HRS)

until between 24 and 32 hours. In the latter case rates of excretion over the first 16 hours were low, and after 32 hours they again diminished gradually and irregularly. In both cases metabolites could still be detected at 96 hours after administration.

For each horse, excretion of individual metabolic groups over successive samples was irregular, and continued in at least one of the groups for the duration of the experiment. Unconjugated metabolites occurred almost completely in the sulphoxide form. Their rate of excretion was low and did not exceed 2.5 mg/hr for any horse, the average rate being approximately 0.5 mg/hr over the 96 hour period. Unconjugated sulphide derivatives were detected in small quantity in only one case, (Horse 4), from approximately 32 hours after injection. On average, unconjugated metabolites accounted for less than 2% of the dose.

Excretion of glucuronide conjugated sulphoxide derivatives also continued for at least 96 hours in five experiments, but one horse (7) showed no trace of such metabolites. Their average rate of excretion over 96 hours was less than 2mg/hr. Glucuronide conjugated sulphide metabolites were much more abundant. However, horse 5 which was dosed twice, only showed trace amounts of such derivatives. The other four horses excreted variable amounts over each experiment and in no case were they detected by 80 hours after injection. Glucuronide conjugates, on average, accounted for approximately 9% of the dose.

Sulphate conjugated metabolites were only detected occasionally

FIGURE 18.

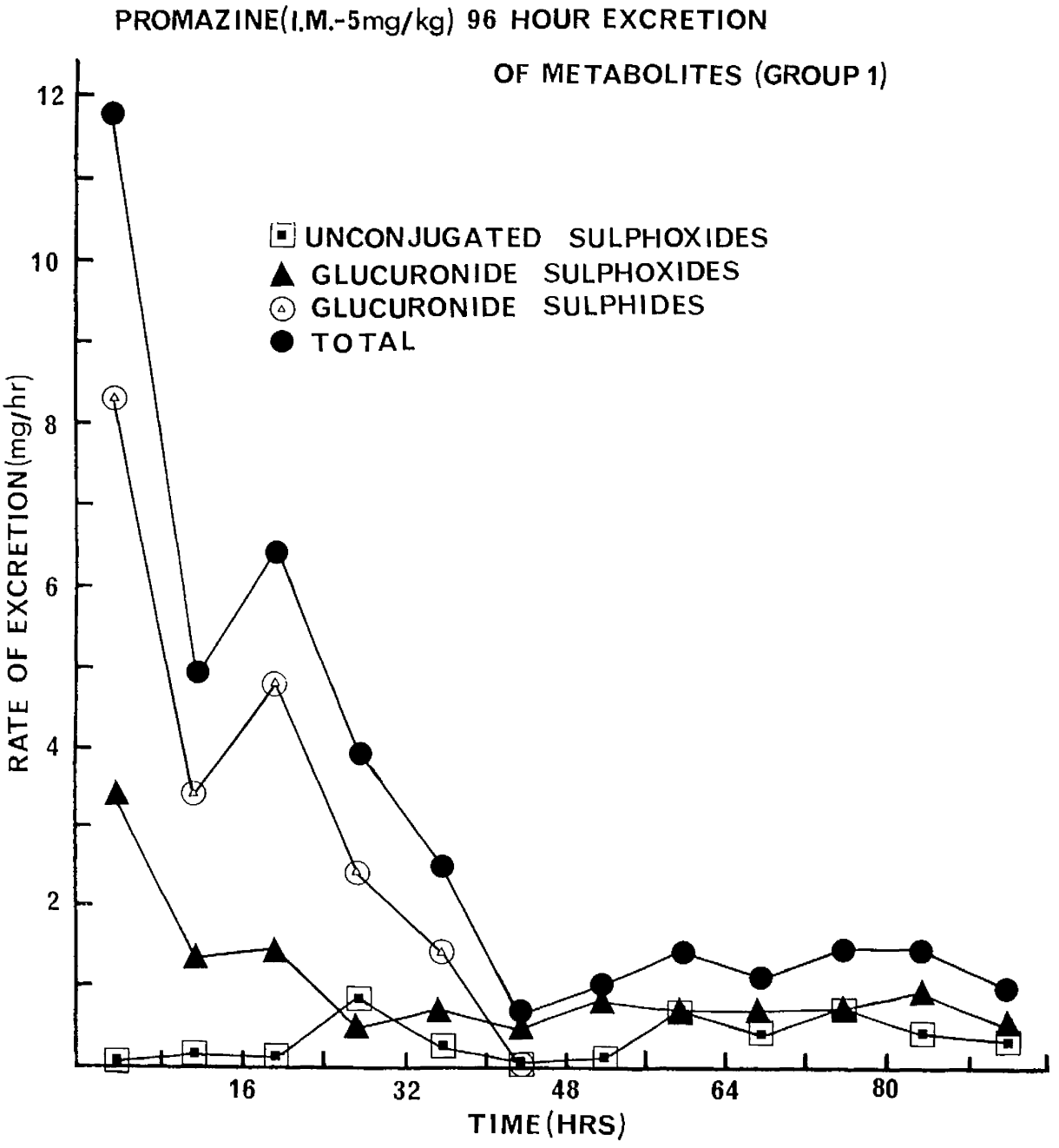
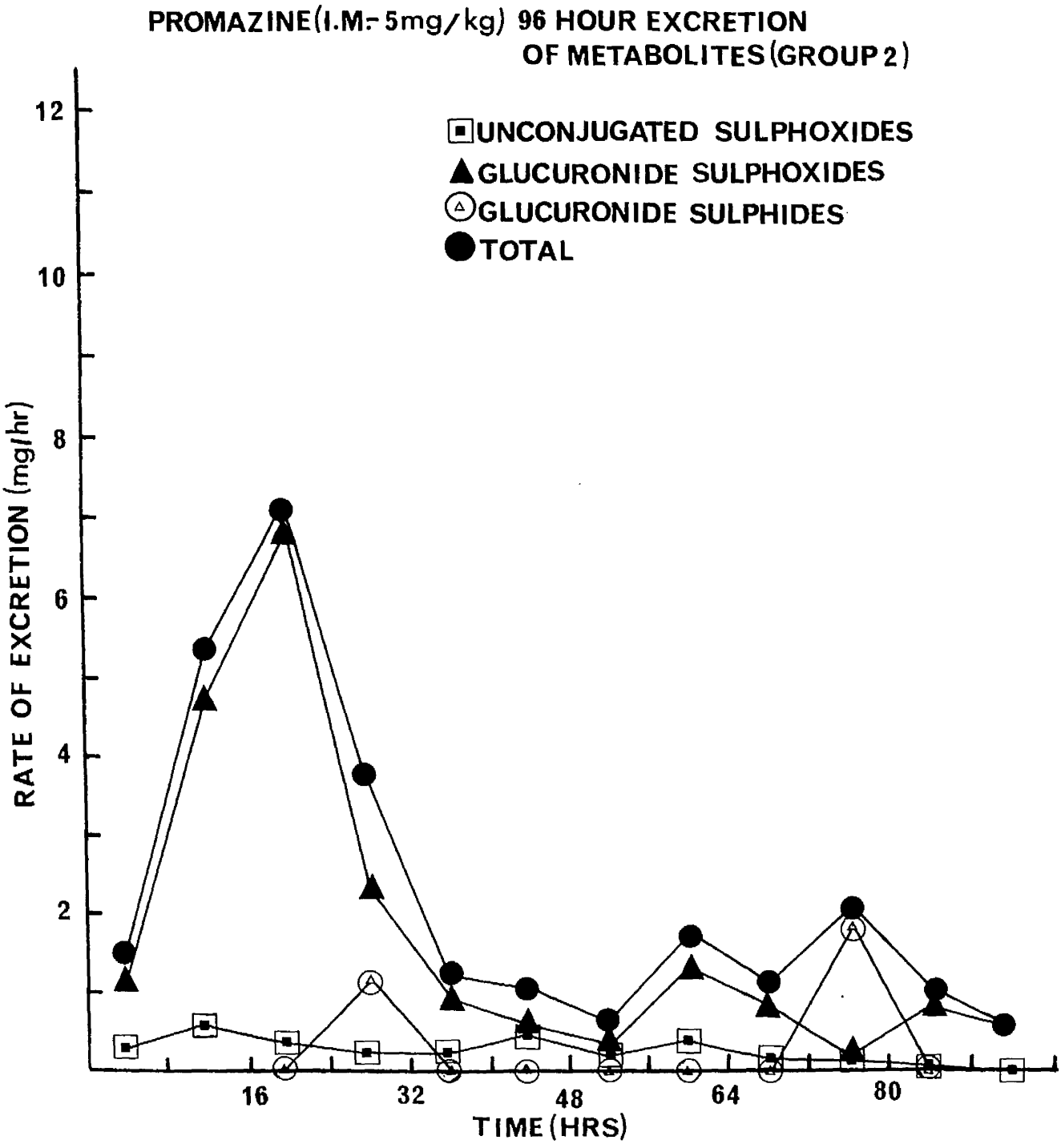

PROMAZINE(I.M.-5mg/kg) 96 HOUR EXCRETION
OF METABOLITES (GROUP 1)
UNCONJUGATED SULPHOXIDES
GLUCURONIDE SULPHOXIDES
GLUCURONIDE SULPHIDES
TOTAL
RATE OF EXCRETION(mg/hr)
12
10
8
6
4
2
16
32
48
64
80
TIME(HRS)

FIGURE 19.


PROMAZINE(I.M- 5mg/kg) 96 HOUR EXCRETION
OF METABOLITES(GROUP 2)
UNCONJUGATED SULPHOXIDES
GLUCURONIDE SULPHOXIDES
GLUCURONIDE SULPHIDES
TOTAL
RATE OF EXCRETION (mg/hr)
12
10
8
6
4
2
16
32
48
64
80
TIME (HRS)

in trace amounts as sulphides after this route of administration. Typical examples of excretion of individual groups of metabolites for the two total types of excretion are shown in Figures 18 and 19.

The percentage of the dose excreted, and values for the separate metabolic fractions are given in Table 5. Glucuronide conjugates predominated in every case, their average ratio to unconjugated metabolites being approximately 5:1. The percentage of dose excreted was low for every horse, the average value being approximately 11%.

(b) Oral Administration

Rates of excretion were plotted for three horses (4, 5, 8) after oral administration of promazine hydrochloride (10 mg/Kg, given as crushed tablets in treacle). The two total excretion patterns described after intramuscular administration were again noted. In one horse, (4), a maximum rate ($\sim$55 mg/hr) was attained within 8 hours, which then decreased rapidly but regularly, excretion being complete by 48 hours. For the other two horses excretion started at a low level and increased gradually to a much less marked maximum ($\sim$25 mg/hr) between 16 and 24 hours after dosing. It then fell off rapidly and irregularly and was complete by around 48 and 72 hours respectively. Patterns of excretion of individual metabolic groups are shown in Figures 20, 21 and 22.

Unconjugated metabolites were again excreted almost entirely as sulphoxides although trace amounts of sulphide derivatives were

FIGURE 20.

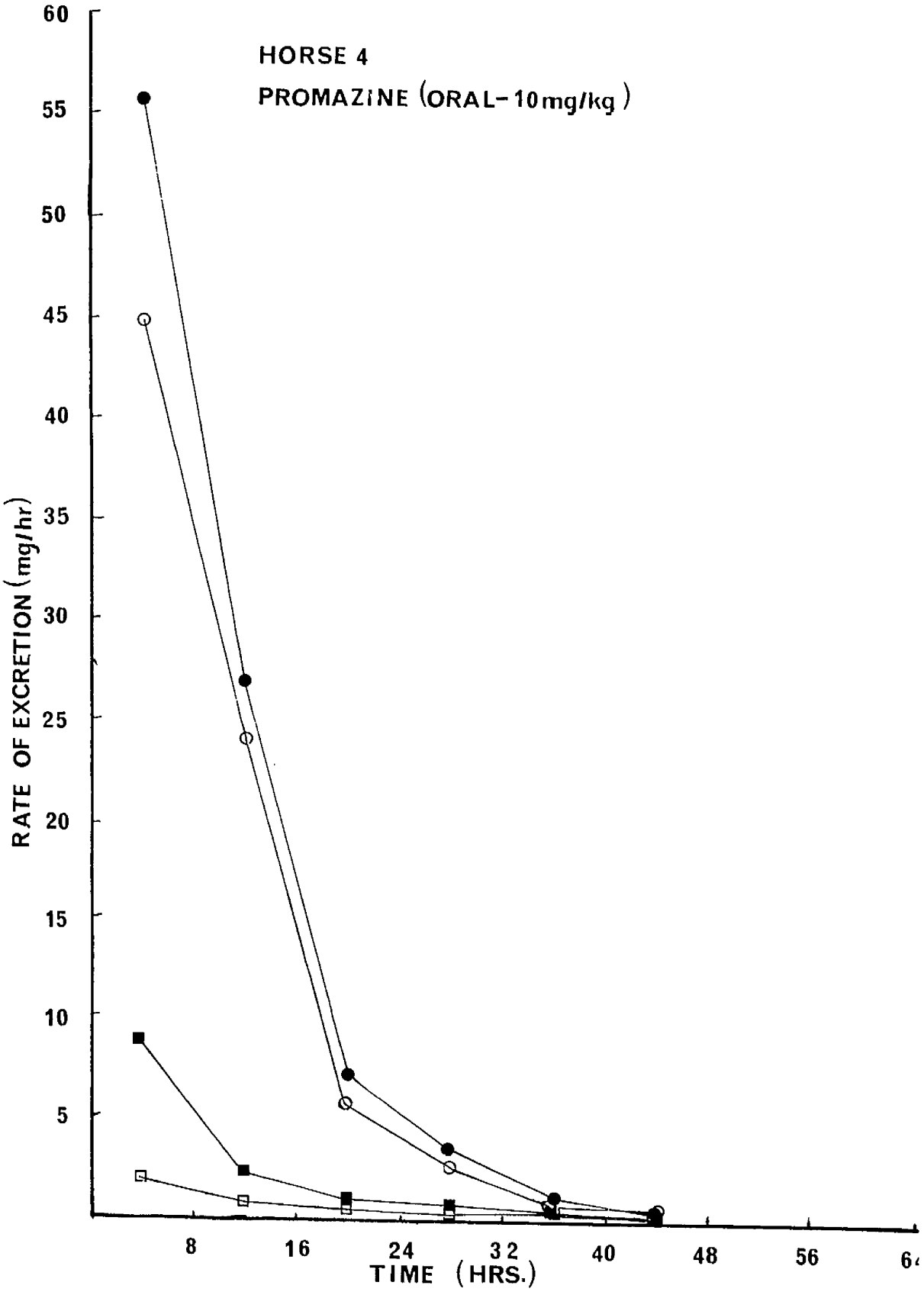

HORSE 4
PROMAZINE (ORAL-10mg/kg)
RATE OF EXCRETION (mg/hr)
60
55
50
45
40
35
30
25
20
15
10
5
8
16
24
32
40
48
56
TIME (HRS.)

FIGURE 21.

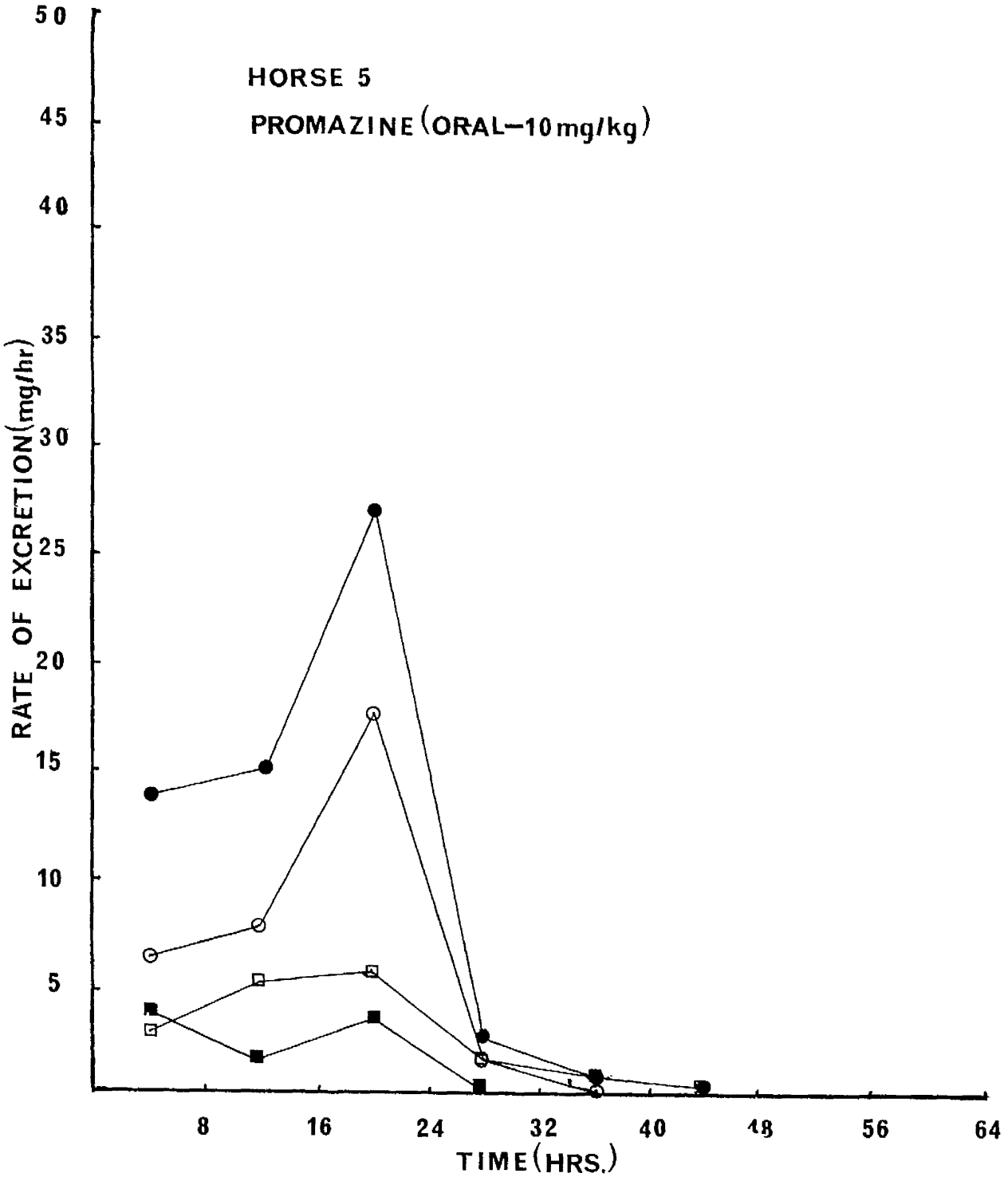
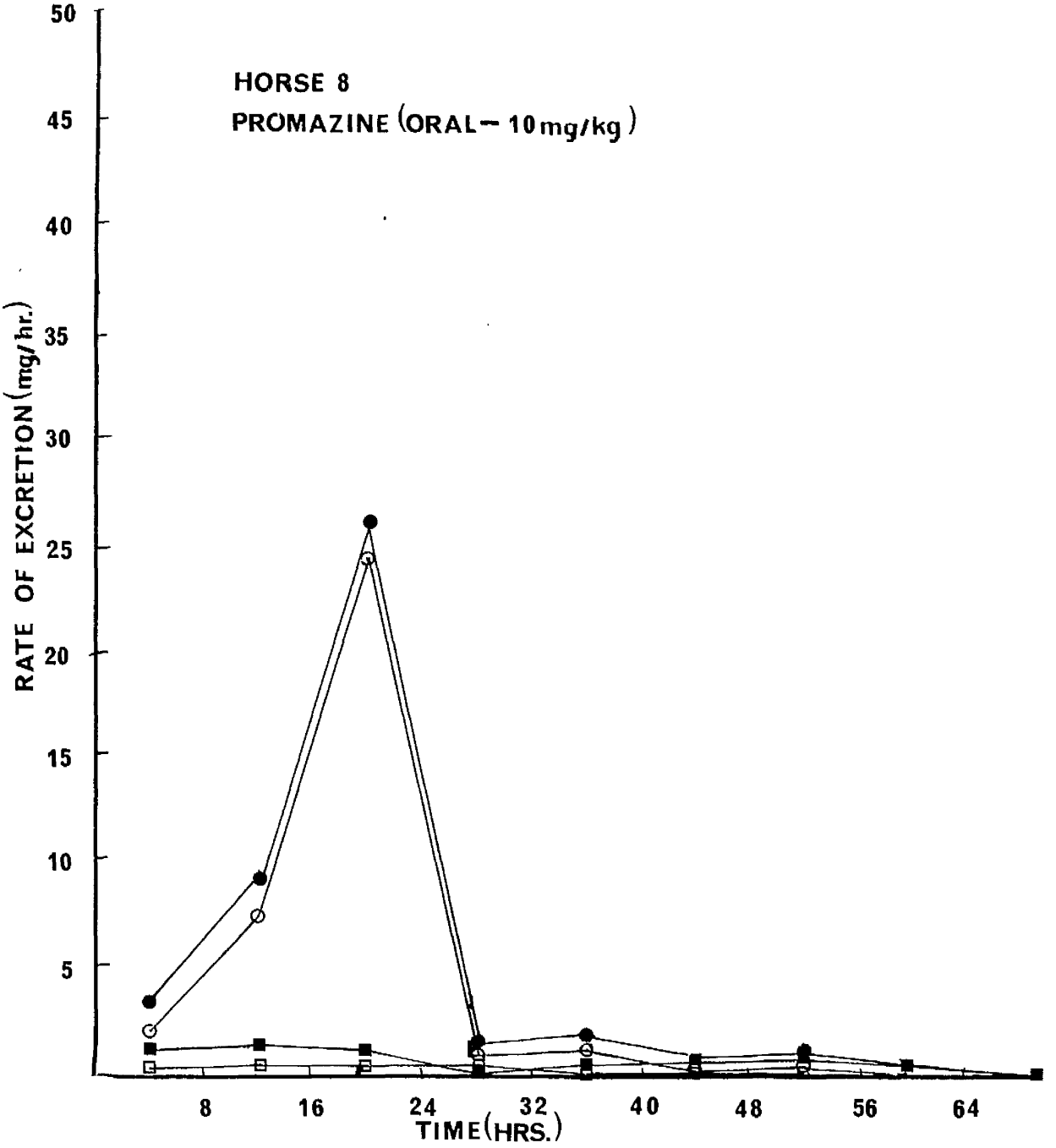

HORSE 5
PROMAZINE (ORAL–10 mg/kg)
RATE OF EXCRETION (mg/hr)
50
45
40
35
30
25
20
15
10
5
8
16
24
32
40
48
56
64
TIME (HRS.)

FIGURE 22.


HORSE 8
PROMAZINE (ORAL – 10 mg/kg)
RATE OF EXCRETION (mg/hr.)
50
45
40
35
30
25
20
15
10
5
8
16
24
32
40
48
56
64
TIME (HRS.)

found from one horse, (4) during the first 24 hours after administration. The percentage of the dose recovered in this fraction was low ($\sim$1%), as were the rates of excretion ($<$6 mg/hr). Excretion of such metabolites was complete by 56 hours for horse 8, and by 48 hours for horses 4 and 5.

Each horse excreted glucuronide conjugates metabolites almost entirely as sulphides, although traces of sulphoxide derivatives were noted in one experiment (Horse 4). Their rate of excretion was also so high compared to the other two fractions that it determined the shape of the total excretion pattern. Horse 4 attained a maximum rate ($\sim$45 mg/hr) within the first 8 hours, whereas maxima were not observed for horses 5 and8 until between 16 and 24 hours after administration. Glucuronide conjugated metabolites, on average, accounted for approximately 7.5% of the dose and were detected until between 40 and 56 hours after dosing.

Sulphates were excreted in similar amounts to unconjugated metabolites, as sulphide derivatives, after this route of administration, although small amounts of the sulphoxide form were excreted by horse 8 between 56 and 72 hours. Their rate remained at a low level and attained 2.5 mg/hr in only three samples during the three experiments. They accounted for approximately 1.5% of the dose.

The percentage urinary excretion, and values for each metabolic fraction are given in Table 5. Glucuronide conjugated

TABLE 5.

The Percentage Urinary Excretion of Dose after Administration of Promazine Hydrochloride to the Horse

Oral Dose (10 mg/Kg)

	Total Excreted	Glucuronides	Sulphates	Unconjugated	Ratio*
Horse 4	13.6	11.2	1.9	0.5	22:4:1
Horse 5	9.3	5.2	1.5	2.6	3:1:2
Horse 8	7.4	6.0	0.9	0.5	12:2:1

Average Excreted = 10%
Average Ratio = 6:1:1

I.M. dose (5 mg/Kg)

	Total Excreted	Glucuronides	Sulphates	Unconjugated	Ratio*
Horse 1	10.9	9.6	Trace	1.3	7:0:1
Horse 2	7.6	7.5	Trace	0.1	63:0:1
Horse 4	21.7	18.1	Trace	3.6	5:0:1
Horse 5a	9.1	5.9	Trace	3.2	2:0:1
Horse 5b	7.1	6.1	Trace	1.0	6:0:1
Horse 7	9.6	7.8	Trace	1.8	4:0:1

Average Excreted = 11%
Average Ratio = 5:0:1

* The ratio indicated represents Glucuronides:Sulphates:Unconjugated Metabolites.

metabolites again predominate, their average ratio to the sulphate conjugated and unconjugated metabolites being 6:1:1. Also greater amounts of sulphate conjugated derivatives were detected after this route than after intramuscular administration.

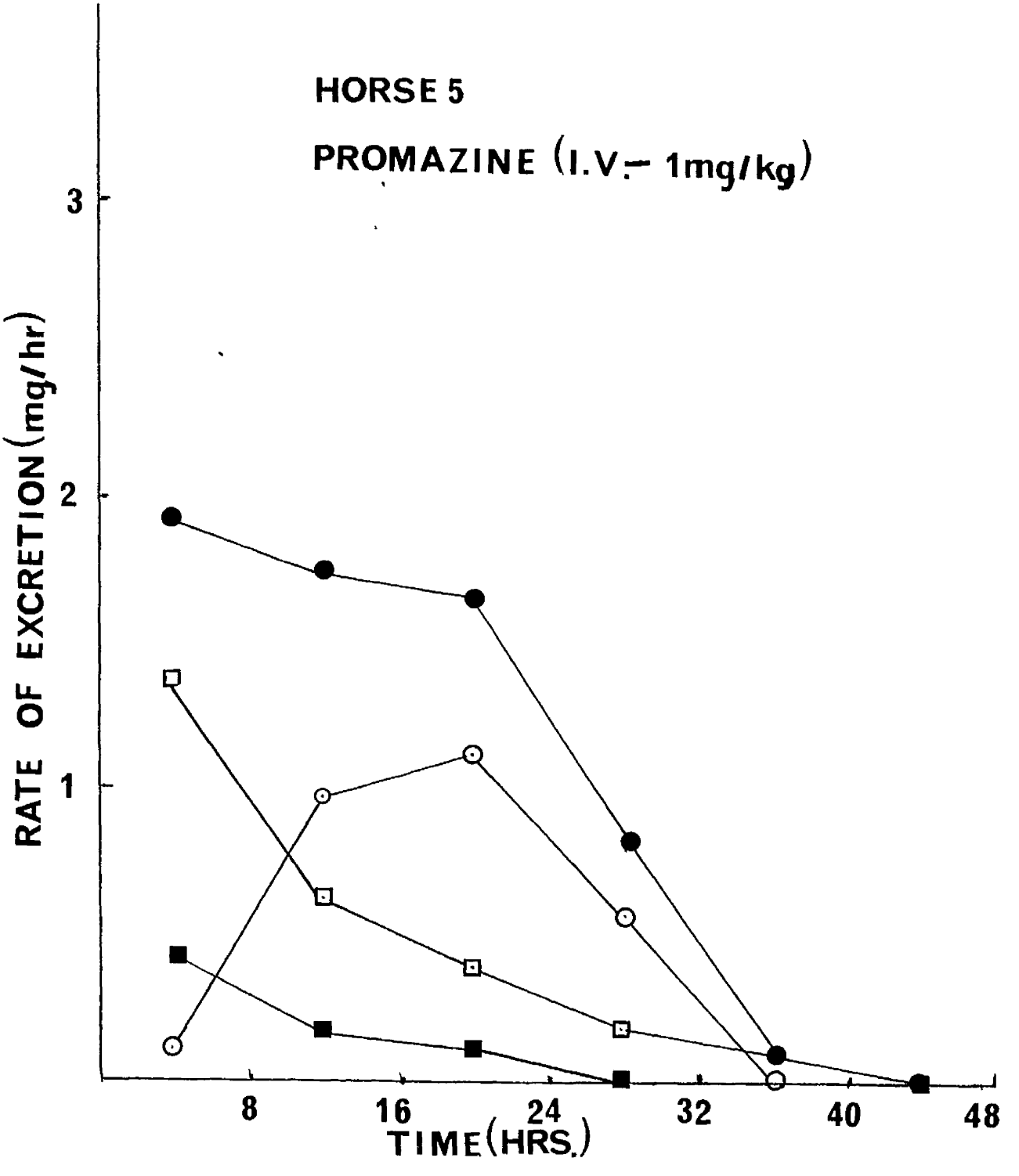
The percentage of dose detected as urinary metabolites was low, having an average value of approximately 10%.

(c) Intravenous Administration.

In addition to oral and intramuscular administration, promazine hydrochloride was also administered intravenously on one occasion to horse 5 (1 mg/Kg as a 5% solution). A graph of the individual and total rates of excretion is shown in Figure 23. The pattern for total metabolites was similar to the first type of excretion noted after intramuscular administration (Pg. 141). The rate reached a maximum (~ 2mg/hr) within 3 hours of injection, and thereafter diminished gradually and regularly reaching zero by 48 hours. Metabolites accounted for approximately 10% of dose.

Unconjugated and glucuronide conjugated metabolites were excreted in approximately equal amounts, each fraction accounting for approximately 4.5% of the dose. Unconjugated derivatives, (all in sulphoxide form), attained a maximum excretion rate (~ 1.5mg/hr within 8 hours which then fell off rapidly, and they were not detected after 48 hours. Glucuronide conjugates were also excreted almost entirely as sulphide derivatives, although sulphoxides were detected in trace amounts between 8 and 24 hours. Their excretion

FIGURE 23.


HORSE 5
PROMAZINE (I.V.- 1mg/kg)
RATE OF EXCRETION (mg/hr)
3
2
1
8
16
24
32
40
48
TIME (HRS.)

reached a maximum (~1mg/hr) between 16 and 24 hours after injection, and was complete by 40 hours.

Much smaller amounts of sulphate conjugated metabolites were detected accounting for approximately 1% of the dose. They existed almost entirely as sulphide derivatives, although traces of the sulphoxide form were excreted during the first 8 hours. Their excretion was complete by 32 hours after injection.

(d) Summary.

(i) After oral and intramuscular administration of promazine total rates of excretion either rapidly attained a maximum value within 8 hours, or slowly rose to a smaller maximum between 24 and 32 hours, diminishing gradually and irregularly in both cases. Intravenous administration,which was carried out only once,produced a maximum rate within the first 8 hours.

(ii) The percentage of dose excreted after all three routes was low, being approximately 10%. Excretion of metabolites was most prolonged after intramuscular administration (> 96 hr.) compared to an average duration of 56 hours for both oral and intravenous routes. Rates of excretion in successive samples were irregular after oral and intramuscular administration, but were more regular after the intravenous route.

(iii) Glucuronide conjugated metabolites predominated after both oral and intramuscular administration, but were present in approximately equal amounts to the unconjugated fraction after the intravenous route. Sulphate conjugated metabolites were detected in only small amounts after all three routes. After oral and

intravenous administration they accounted for approximately 1% of the dose, whereas intramuscular injection produced only trace amounts.

(iv) Unconjugated metabolites were detected almost entirely as sulphoxides, irrespective of route. Conjugated metabolites on the other hand, consisted predominantly of sulphide derivatives.

(2) Chlorpromazine

(a) Intramuscular Administration.

Rates of excretion over three experiments, (Horses 4, 5a, 5b), were plotted after intramuscular administration of chlorpromazine hydrochloride (2mg/Kg as a 5% solution). However, neither of the total excretion patterns noted after intramuscular administration of promazine (Pg.141) was immediately obvious. On both occasions horse 5 attained a slight maximum rate between 24 and 40 hours after injection, (1.5 mg/hr between 24 and 32 hours for horse 5a and 2 mg/hr between 32 and 40 hours for horse 5b) and metabolites were detected until $\sim$ 64 hours. Horse 4 attained a much higher rate of excretion. It rose to a maximum of 5.7 mg/hr between 16 and 24 hours after administration, and excretion was not complete until 96 hours.

Glucuronide conjugated metabolites again appeared in greatest concentration, and were the biggest contributing factor to the total excretion pattern. Horse 5, on both occasions, excreted this fraction almost entirely as sulphoxide derivatives, although traces of sulphide metabolites were also detected. It excreted

glucuronides until ~ 64 hours after injection, and, with the exception of one sample, their rate did not exceed 1 mg/hr. Horse 4, on the other hand, excreted glucuronides entirely in the sulphide form up to 96 hours after injection. The rate attained two maxima, of the order of 6 mg/hr, between 16 and 48 hours. Glucuronide conjugated metabolites on average accounted for approximately 8% of the dose.

Unconjugated metabolites were excreted entirely as sulphoxide derivatives, and in all three experiments their rate of excretion was less than 0.5 mg/hr. This fraction was detected from horse 4 for the duration of the experiment, whereas they were not excreted by horse 5 after 64 hours. Samples voided by horse 5a during the first 16 hours contained no trace of unconjugated metabolites, but they were subsequently detected until 56 hours after administration. Such derivatives were responsible for approximately 1.2% of the dose.

In all three experiments only trace amounts of sulphate conjugated metabolites were found almost entirely as sulphides. Excretion patterns of individual metabolites are shown in Figures 24, 25 and 26.

Table 6 shows percentages of dose excreted as individual metabolic groups and once more bears out the predominance of glucuronide conjugates. Their average ratio to unconjugated metabolites was approximately 7:1. The total percentage excreted was also low at 10%. An interesting feature of the results is the considerably higher rate of excretion of glucuronides by horse

FIGURE 24.

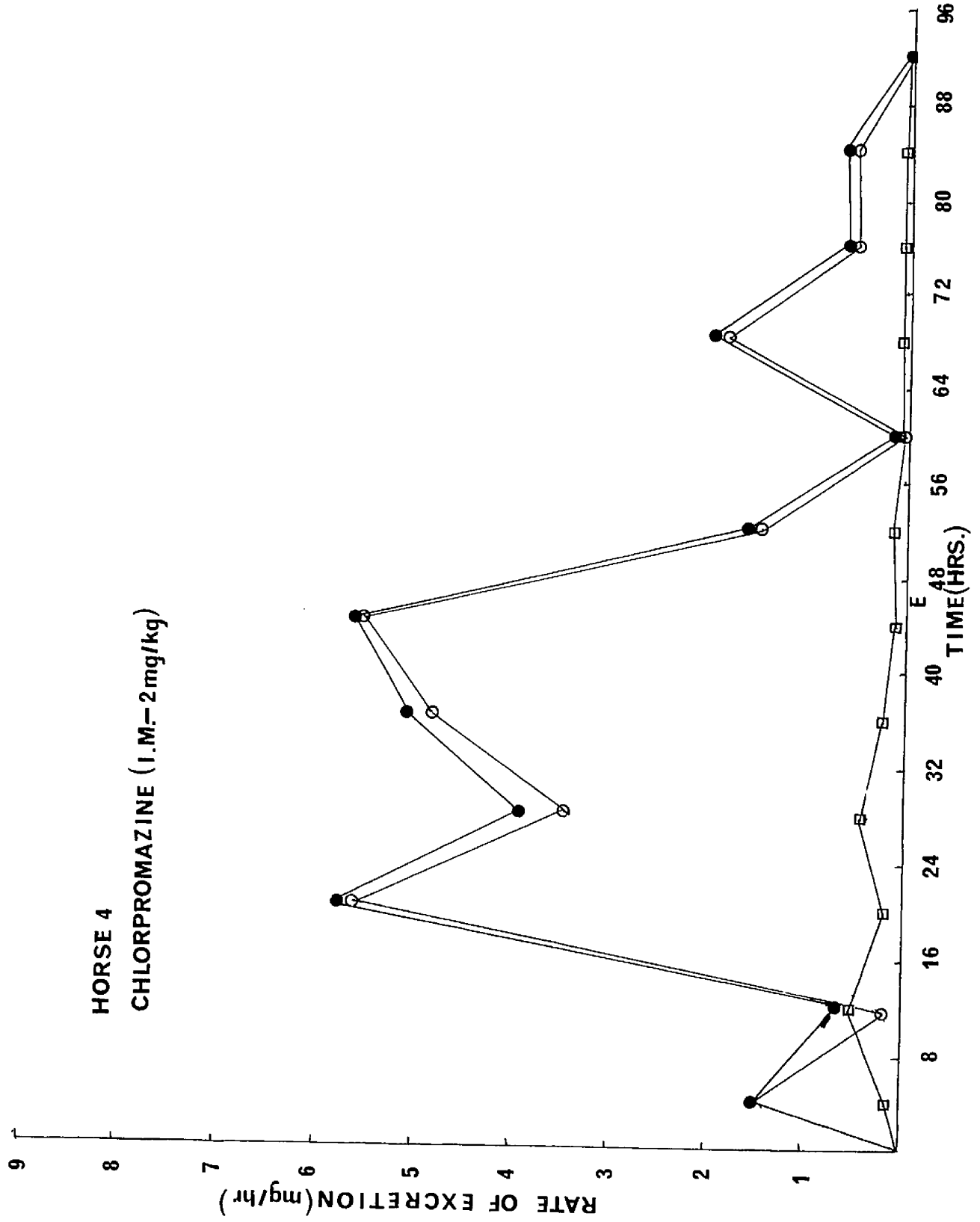

HORSE 4
CHLORPROMAZINE (I.M.–2mg/kg)
RATE OF EXCRETION(mg/hr)
TIME(HRS.)
E
1
2
3
4
5
6
7
8
9
8
16
24
32
40
48
56
64
72
80
88
96

FIGURE 25.

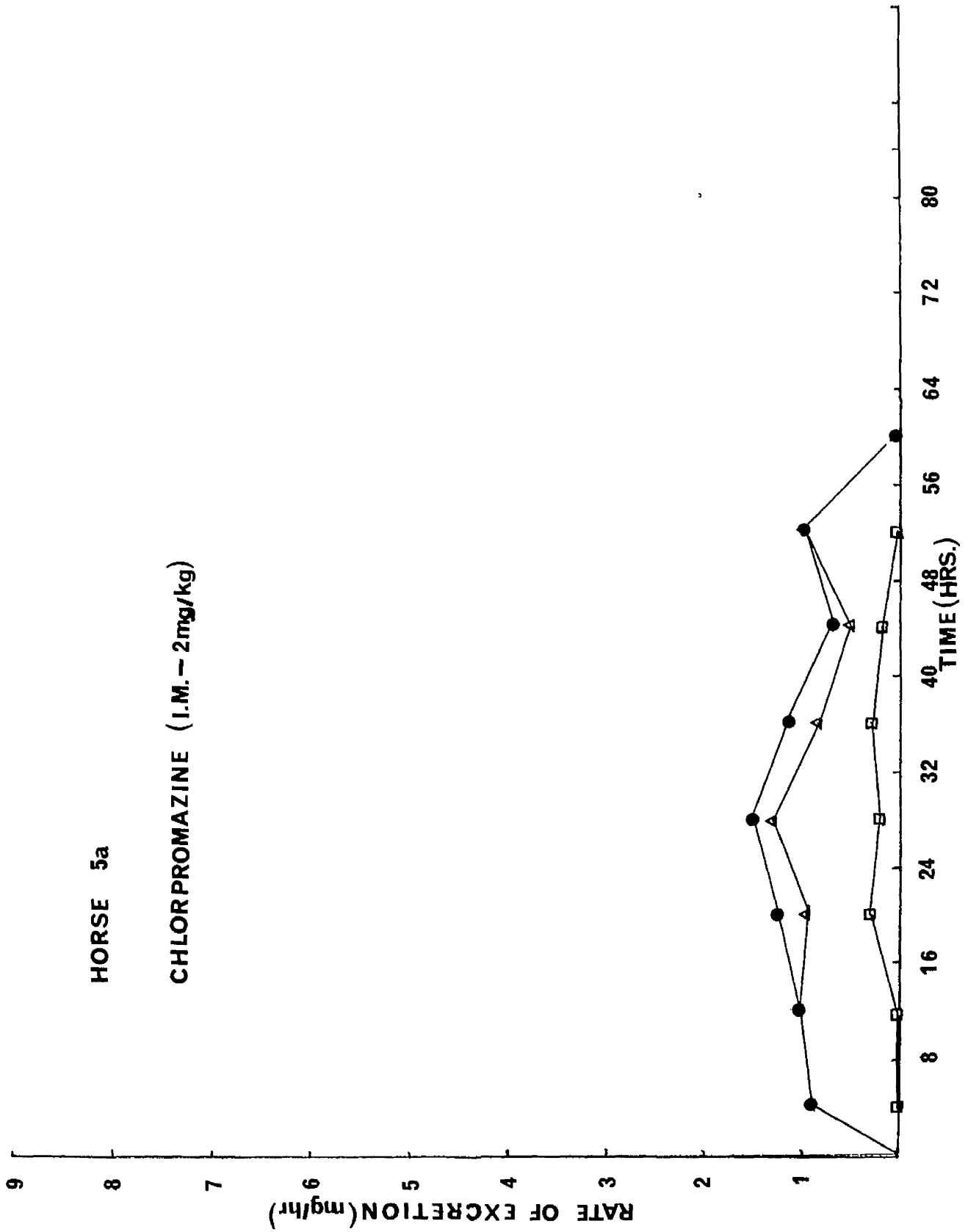
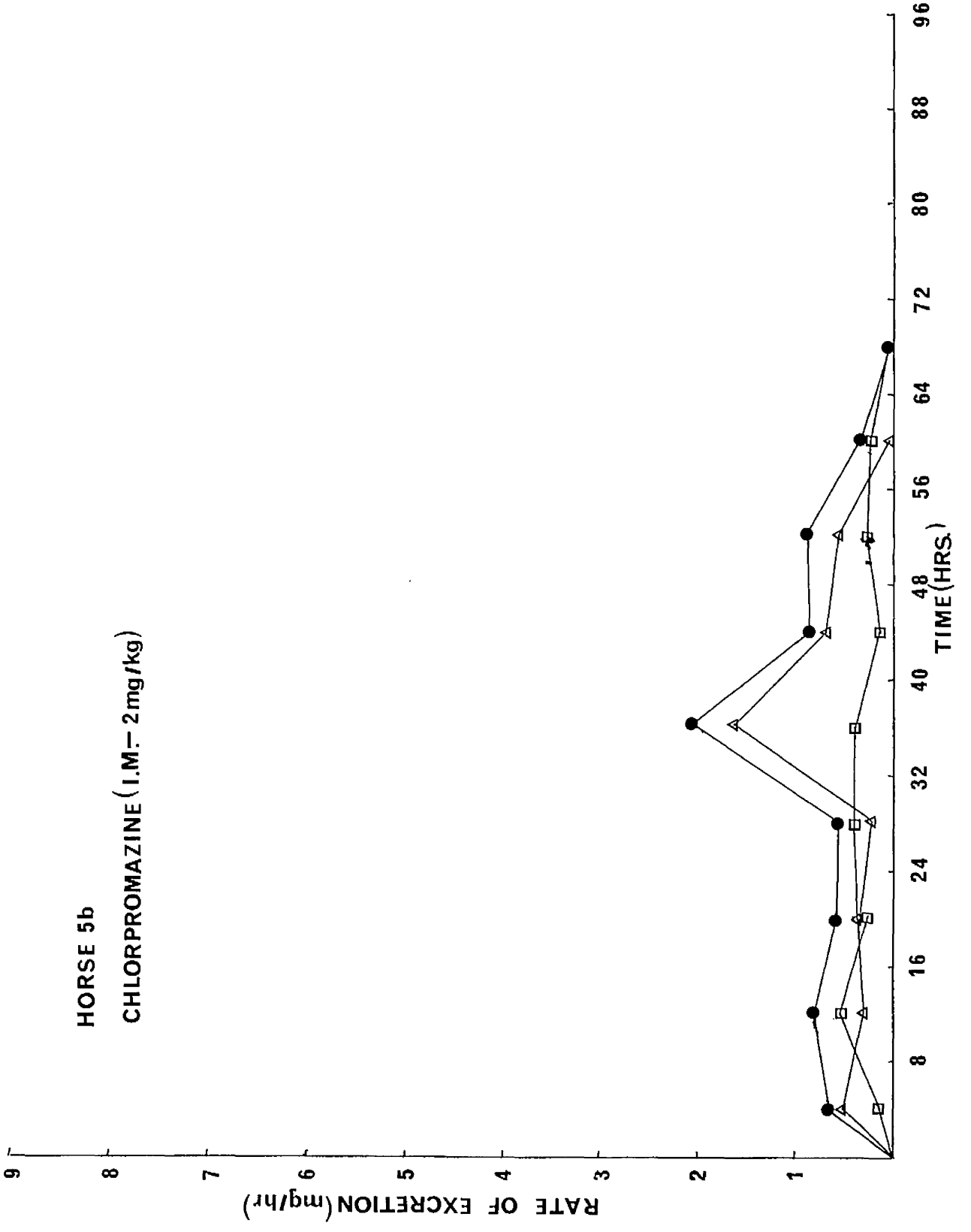

HORSE 5a
CHLORPROMAZINE (I.M. – 2mg/kg)
RATE OF EXCRETION (mg/hr)
TIME (HRS.)
1
2
3
4
5
6
7
8
9
8
16
24
32
40
48
56
64
72
80

FIGURE 26.


HORSE 5b
CHLORPROMAZINE (I.M.– 2mg/kg)
RATE OF EXCRETION (mg/hr)
TIME (HRS.)
1
2
3
4
5
6
7
8
9
8
16
24
32
40
48
56
64
72
80
88
96

4 compared to the other animals, which was also noted after administration of promazine, (Pg. 146)

(b) Oral Administration.

Chlorpromazine hydrochloride, (5 mg/Kg, given as crushed tablets in treacle), was administered orally to horses 5 and 7, the latter being dosed twice, and excretion of metabolites was followed over 96 hours. Two types of total excretion were obtained similar to those experienced with promazine. Horse 5 attained a maximum rate ($\sim$35 mg/hr) within 8 hours which then fell off gradually and irregularly, and no metabolites were detected after 80 hours. Horse 7, on the first occasion, attained a maximum rate (31.6 mg/hr) between 8 and 16 hours after administration, with a second maximum ($\sim$30 mg/hr) between 24 and 32 hours. In the second experiment the maximum also occurred between 24 and 32 hours but was much smaller (14.8 mg/hr). For this horse the lengths of excretion were 96 and 80 hours respectively. Individual excretion patterns for each of the experiments are shown in Figures 27, 28 and 29.

Glucuronide conjugated metabolites, again forming the main fraction, were the deciding factor in the shape of the total excretion pattern. They were present almost entirely in the sulphide form although small amounts of sulphoxide derivatives were detected from horse 5. For all three experiments glucuronides were excreted for longer than any other group (80-96hr) and, on average, accounted for 23% of the dose.

The unconjugated fraction were excreted almost entirely as sulphoxides, although small amounts of sulphide derivatives were

FIGURE 27.

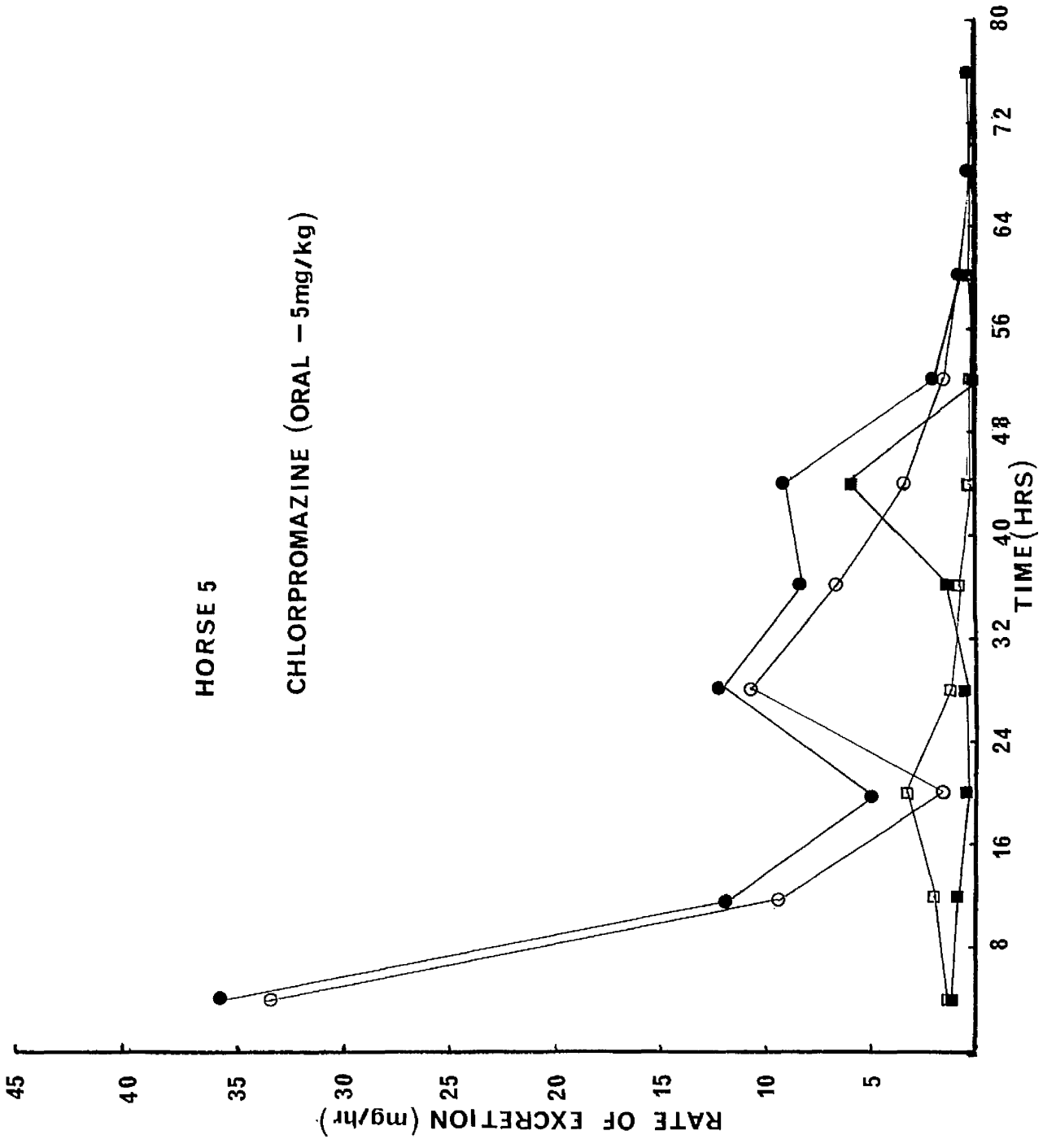

HORSE 5
CHLORPROMAZINE (ORAL – 5mg/kg)
RATE OF EXCRETION (mg/hr)
TIME (HRS)
45
40
35
30
25
20
15
10
5
8
16
24
32
40
48
56
64
72
80

FIGURE 28.

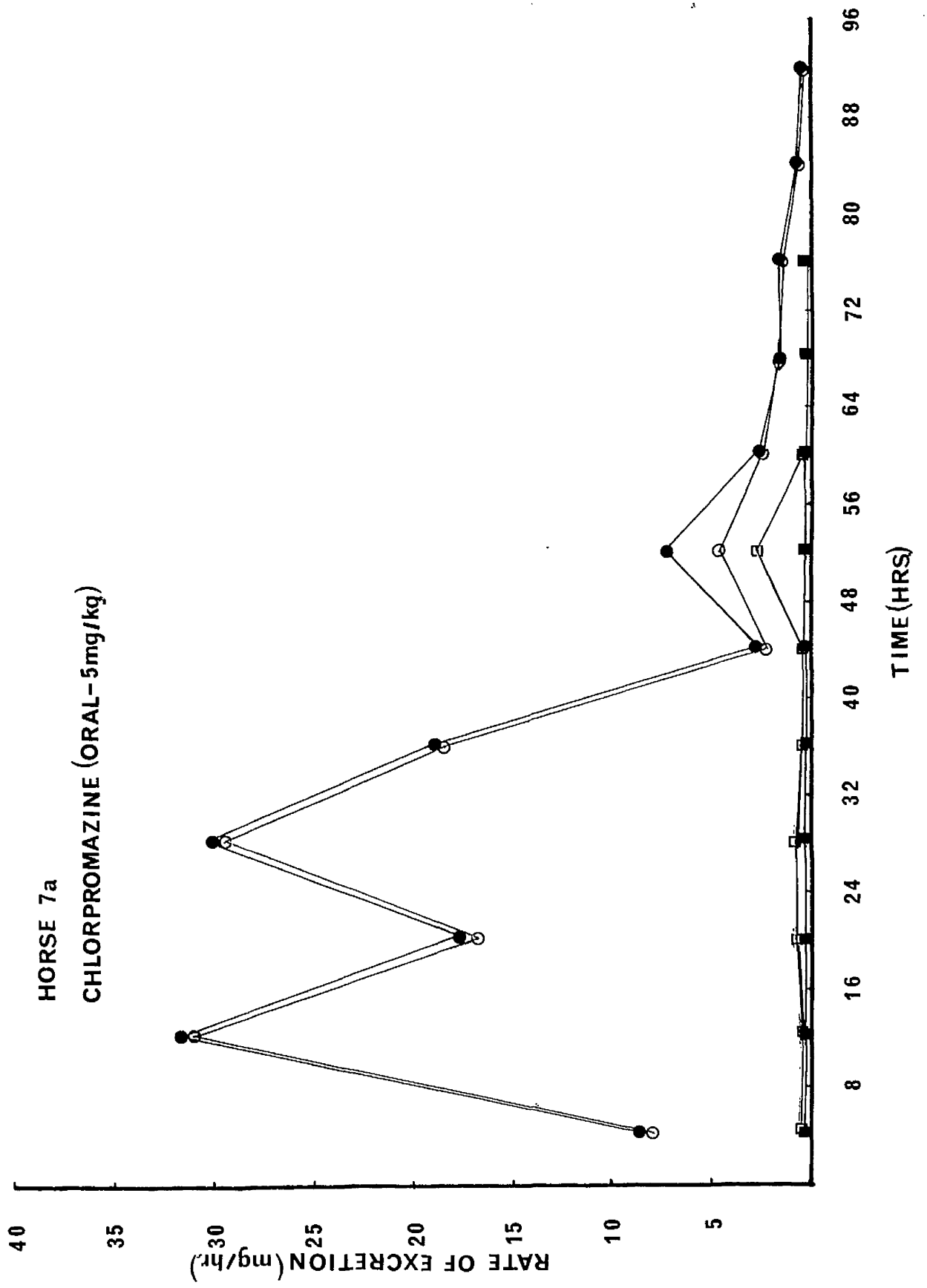

HORSE 7a
CHLORPROMAZINE (ORAL-5mg/kg)
RATE OF EXCRETION (mg/hr)
40
35
30
25
20
15
10
5
8
16
24
32
40
48
56
64
72
80
88
96
TIME (HRS)

FIGURE 29.

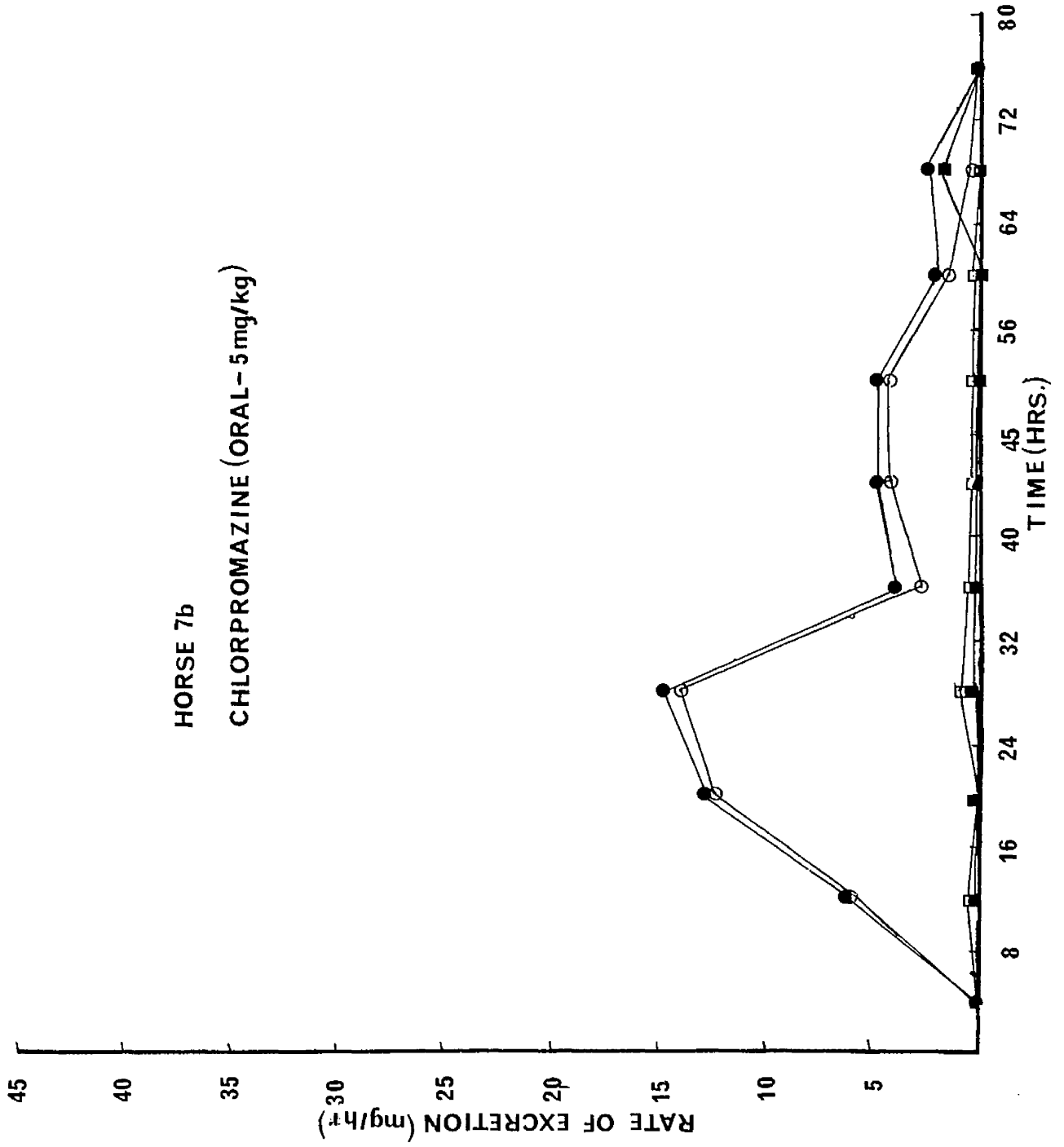

HORSE 7b
CHLORPROMAZINE (ORAL-5mg/kg)
RATE OF EXCRETION (mg/hr)
TIME (HRS.)
45
40
35
30
25
20
15
10
5
8
16
24
32
40
45
56
64
72
80

TABLE 6

The Percentage Urinary Excretion of Dose after Administration of Chlorpromazine Hydrochloride to the Horse

Oral Dose (5 mg/Kg)

	Total Excreted	Glucuronides	Sulphates	Unconjugated	Ratio *
Horse 5	24.3%	19%	2.8%	2.5%	7:1:1
Horse 7a	39.5%	37.5%	0.3%	1.7%	22:0.2:1
Horse 7b	15.6%	13.6%	0.9%	1.1%	16:1:1

Average Excreted = 27%
Average Ratio = 18:1:1

I.M. Dose (2 mg/Kg)

	Total Excreted	Glucuronides	Sulphates	Unconjugated	Ratio *
Horse 4	18.5%	17.2%	Trace	1.3%	13:0:1
Horse 5a	5.5%	4.8%	Trace	0.7%	7:0:1
Horse 5b	4.6%	3.0%	Trace	1.7%	2:0:1

Average Excreted = 10%
Average Ratio = 7:0;1

* The ratio indicated represents Glucuronides:Sulphate:Unconjugated metabolites

detected from horse 7 between 48 and 64 hours. Their rate of excretion never exceeded 3 mg/hr and they were not found after 72 hours from either horse.

Sulphate conjugated metabolites were present in amounts similar to the unconjugated fraction, ($\sim$ 1.5%), almost entirely as sulphides, although small amounts of sulphoxide derivatives were occasionally detected. With the exception of one sample their rate of excretion was less than 2.5 mg/hr, and was complete by between 72 and 80 hours.

Table 6 shows the percentages of dose excreted as individuaal metabolic groups. Glucuronide conjugated metabolites again formed the major fraction having a ratio to the other groups of approximately 18:1:1. A greater percentage of dose was recovered after this route than after intramuscular administration of chlorpromazine, accounting for approximately 27%.

(c) Summary

(i) Both types of total excretion pattern noted after administration of promazine were noted after oral administration of chlorpromazine. However only the second type was found after the intramuscular route. The percentage of the dose excreted was low being approximately 10% after intramuscular injection, and 27% for the oral route.

(ii) Glucuronide conjugated metabolites were the predominating fraction occurring mostly as sulphide derivatives. In one horse this group was excreted predominantly in sulphoxide form, but this seems to be a metabolic abnormality peculiar to the horse (Pg. 187).

Sulphate conjugated metabolites also were found almost entirely as sulphides and unconjugated metabolites as sulphoxides. Only trace amounts of sulphates were detected after intramuscular administration, whereas they accounted for approximately 1.5% of the dose after the oral route. The ratios of glucuronides to sulphates to unconjugated metabolites after oral and intramuscular administration were 18:1:1 and 7:0:1 respectively.

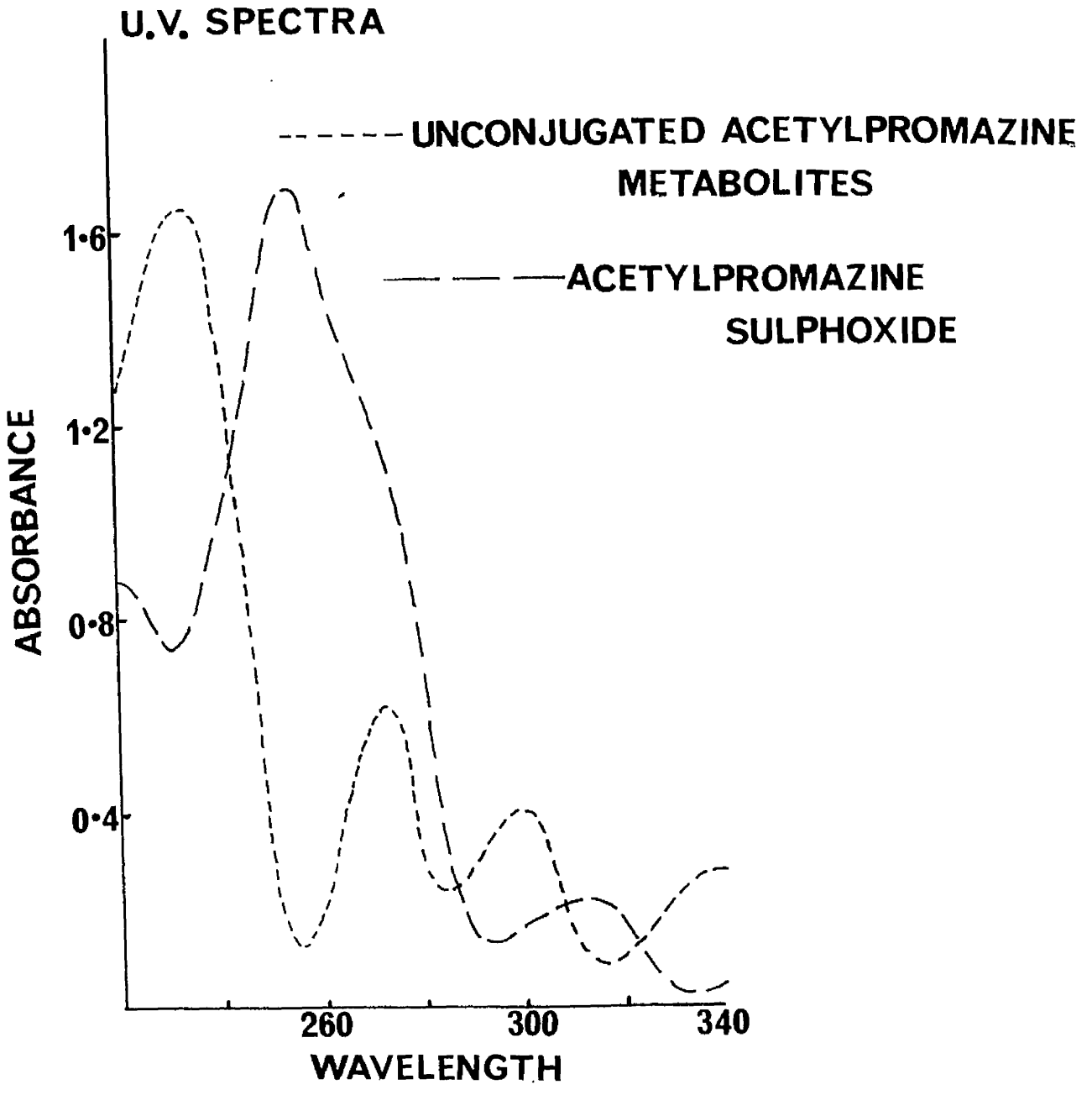
(iii) As with promazine, excretion of chlorpromazine was prolonged and irregular, lasting up to 96 hours after oral administration and 64 hours after the intramuscular route.

(3) Acepromazine and Propionylpromazine.

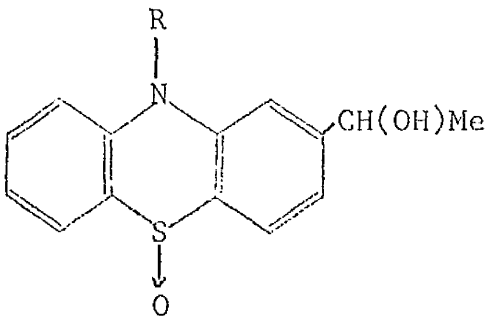
As previously described, (Pg.105) phenothiazine derivatives having a ketone group in conjugation with the aromatic ring, (i.e. acepromazine and propionylpromazine), have different ultra violet spectra to those of derivatives lacking this combination, (i.e. promazine, chlorpromazine, etc.) The former compounds have two absorbance maxima at approximately 240 and 280 mμ, and their corresponding sulphoxides produce two peaks at ~ 250 and 310 mμ. The latter, on the other hand, absorb at ~250 and 300 mμ, and their sulphoxides produce four peaks at approximately 230, 270, 300 and 340 mμ.

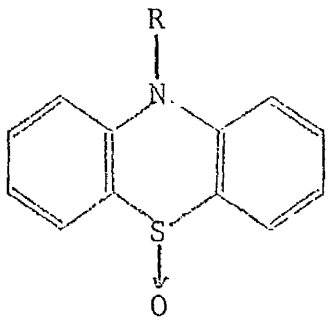
During initial studies on acepromazine, extracts of metabolites produced neither of the spectra described for derivatives of this compound, but unconjugated metabolites had ultra violet spectra with four peaks at 230, 270, 300 and 340 mμ corresponding to that of sulphoxide derivatives of promazine (Figure 30). This suggested

FIGURE 30.


U.V. SPECTRA
UNCONJUGATED ACETYLPROMAZINE METABOLITES
ACETYLPROMAZINE SULPHOXIDE
ABSORBANCE
1·6
1·2
0·8
0·4
260
300
340
WAVELENGTH

that such derivatives have either lost the side chain ketone group at the 2-position completely, or that this substituent has been reduced to the corresponding secondary alcohol, accompanied in either case by oxidation to the sulphoxide form. The general formulae of such products are:





Since both these compounds give ultra violet spectra similar to promazine sulphoxide, it was decided to assay unconjugated metabolites of acepromazine as sulphoxides of promazine. Similar results were subsequently obtained for unconjugated metabolites of propionylpromazine, which were also determined in this manner. Ultra violet spectra of the conjugated fractions from acepromazine resembled neither those of promazine or acepromazine. Instead, successive extracts produced maximum absorbance at varying wavelengths, typical of the situation previously described (Pg. 112) where different ratios of acepromazine and its sulphoxide in solution produce variations in the wavelengths of the major peak.

Due to this, conjugated fractions were assayed from the visible spectra of their respective extracts after colour formation with sulphuric acid (Pg. 104). Concentrations were read from standard graphs of concentration plotted against absorbance prepared from the

visible spectra of standard solutions of acepromazine treated with sulphuric acid. Conjugated metabolites of propionylpromazine were also assayed in this manner.

(a) Intramuscular Administration.

Excretion patterns were plotted after intramuscular administrati of acepromazine maleate, (0.5 mg/Kg as a 1% solution), to horses 4, 5 and 8. Also, to confirm that the excretion data, and metabolic routes found for acepromazine were typical of phenothiazine derivatives with a conjugated ketone grouping, a similar dose of propionylpromazine (as the phosphate) was subsequently administered to horse 7. Urine collections in this case were made twice daily for 5 days after injection, and 100 ml aliquots from each sample were analysed.

For both drugs, unconjugated metabolites predominated in the form of sulphoxide derivatives of promazine. The rates of excretion after administration of acepromazine were low, being less than 0.25 mg/hr throughout the three experiments, and were not so irregular as with promazine or chlorpromazine. Two types of total excretion pattern were again obtained. Horses 4 and 8 attained a maximum rate ($\sim$0.2 mg/hr) within 8 hours of injection, whereas for horse 5 the rate rose to a slight maximum (0.25 mg/hr) between 8 and 16 hours. In all three cases excretion then tailed off gradually and was complete by 32 hours. Excretion patterns for the three horses are shown in Figure 31. Metabolites of propionylpromazine, almost entirely as unconjugated derivatives, were readily detected up to 54 hours after injection and were

FIGURE 31.

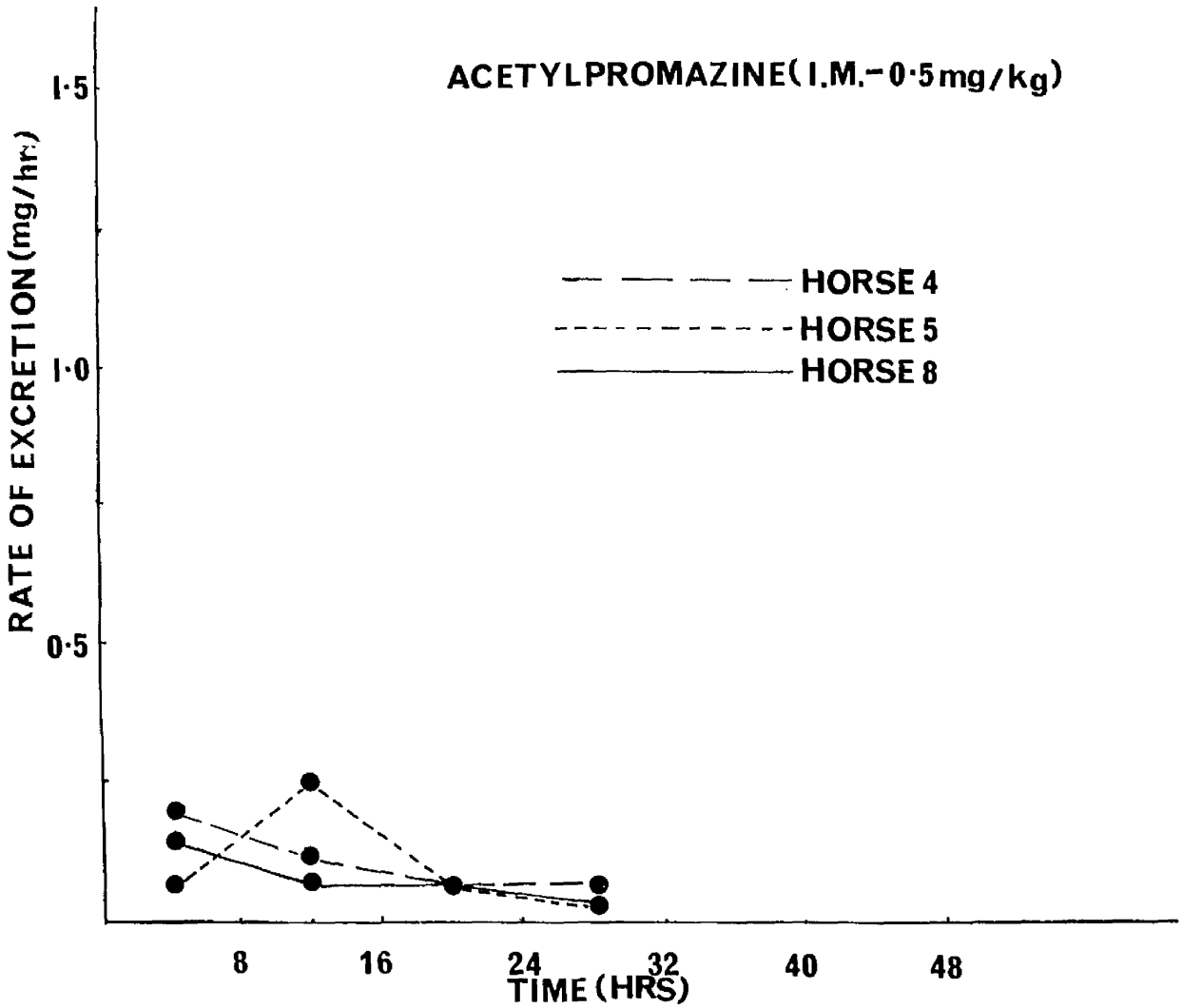

ACETYLPROMAZINE(I.M.-0·5mg/kg)
HORSE 4
HORSE 5
HORSE 8
RATE OF EXCRETION(mg/hr.)
1·5
1·0
0·5
8
16
24
32
40
48
TIME (HRS)

Table 7 Amount Excreted, Urinary Volume and pH after administration of propionylpromazine phosphate to the horse.

Sample	Time(Hrs)	Vol(ml)	pH	Unconjugated(mg)	Glucuronides(mg)	Sulphate(mg)
1	22	680	7.7	5.5 mg	Trace	Trace
2	26	670	7.7	0.3 mg	Trace	Trace
3	51	640	7.8	0.3 mg	Trace	Trace
4	54	700	7.8	0.4 mg	Trace	Trace
5	75	1000	7.8	Trace	Trace	Trace
6	94	850	7.3	-	Trace	Trace

present in trace amounts at 75 hours. Table 7 gives data concerning the concentrations found in each sample after administration of this drug.

Glucuronide and sulphate conjugated metabolites of both drugs were present in only trace amounts, indicating that metabolites remain unconjugated after this route of administration. Table 8 shows the percentage of the dose excreted in each of the metabolic groups. Once more, percentages were low, the average for acepromazin being 1.3%.

(b) Oral Administration.

Acepromazine maleate (1 mg/Kg, given as crushed tablets) was administered orally to horses 5 and 8, the former being dosed on two occasions. On plotting the total rates of excretion against time, only the first type of pattern obtained after promazine administration (Pg. 141) was noted, with slight maxima of between 0.5 and 1 mg/hr during the first 8 hours. However, both types of excretion were found for unconjugated metabolites, which again predominated. In this fraction horses 5a and 8 attained maximum rates, ($\sim$0.6 and 0.4 mg/hr respectively), between 8 and 16 hours whereas horse 5b achieved a slight maximum ($\sim$0.4 mg/hr) within 8 hours. Excretion patterns for individual groups of metabolites are shown in Figures 32, 33 and 34.

The unconjugated fraction was again excreted as sulphoxide derivatives of promazine and, with the exception of one sample, their rate did not exceed 0.5 mg/hr throughout the three experiments.

FIGURE 32.

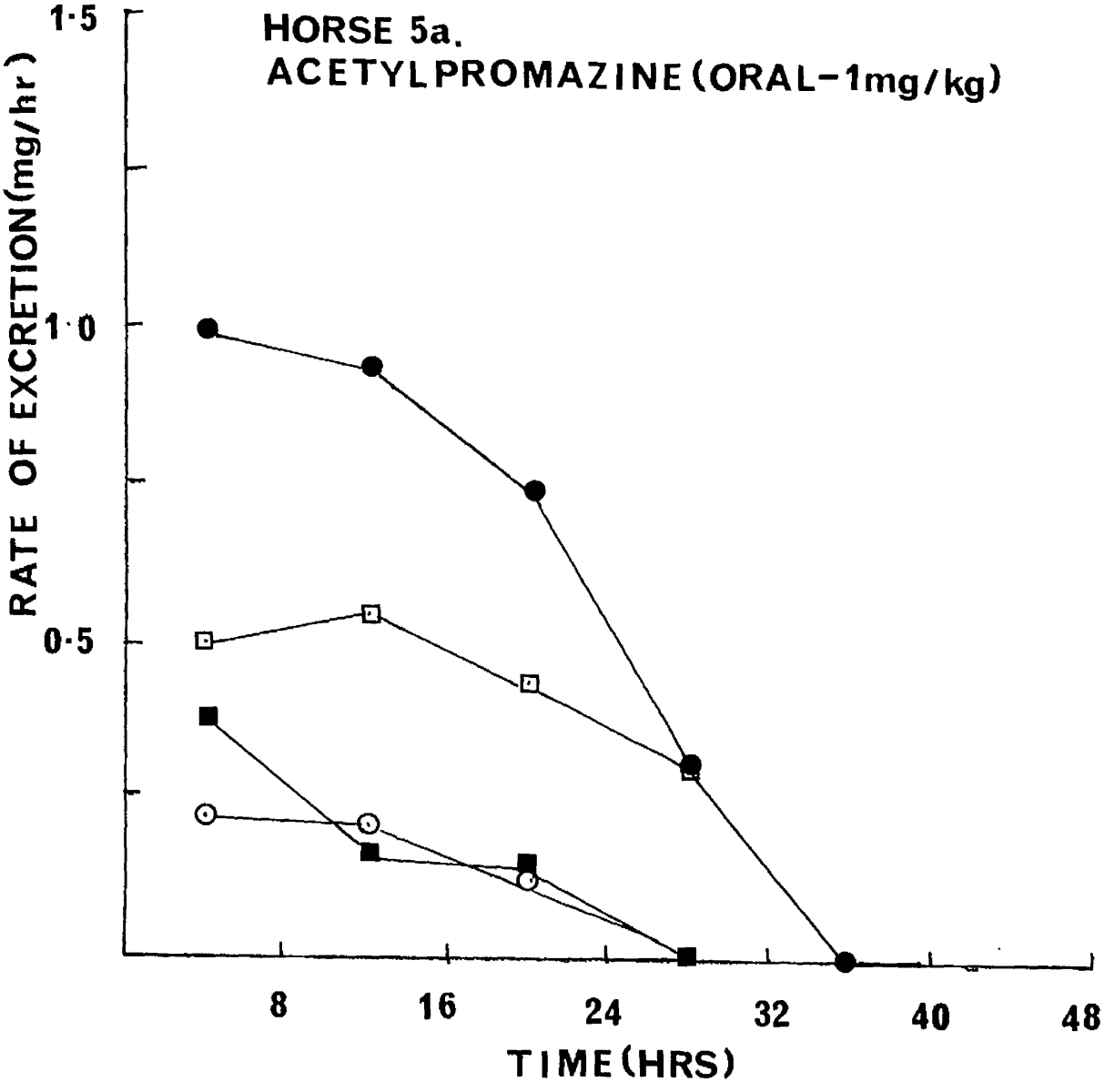

HORSE 5a.
ACETYLPROMAZINE (ORAL-1mg/kg)
RATE OF EXCRETION (mg/hr)
1·5
1·0
0·5
8
16
24
32
40
48
TIME (HRS)

FIGURE 33.

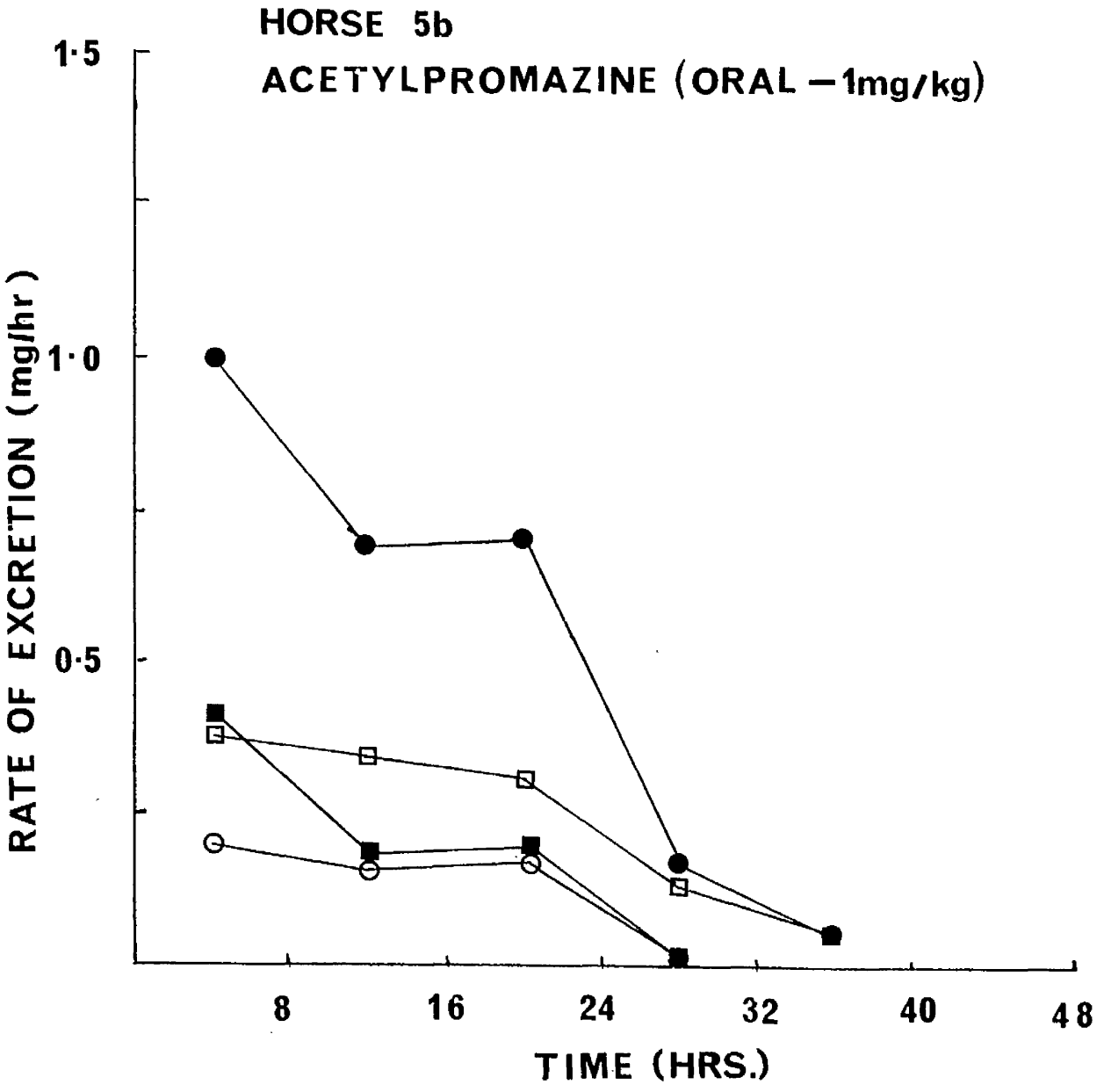
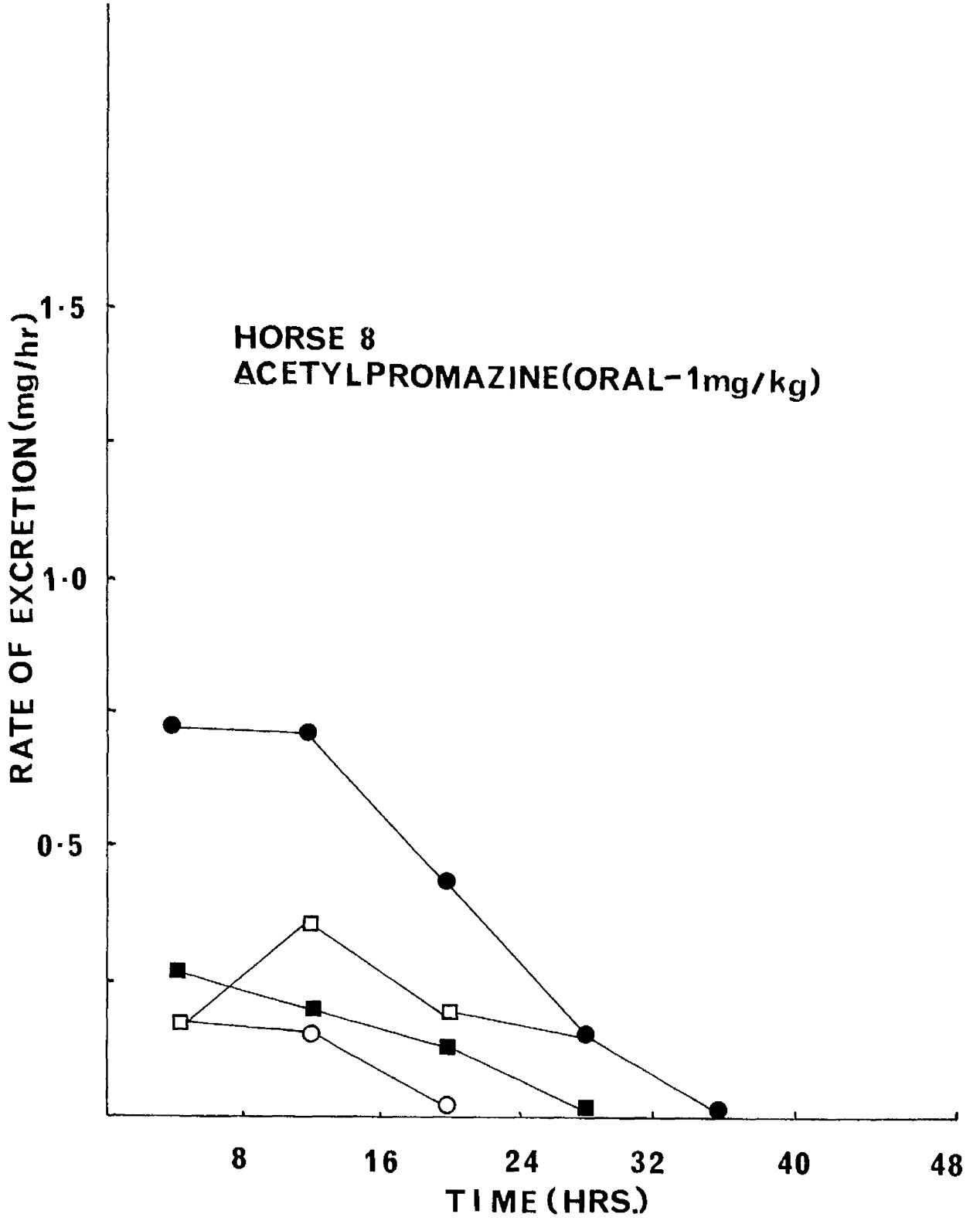

HORSE 5b
ACETYLPROMAZINE (ORAL – 1mg/kg)
RATE OF EXCRETION (mg/hr)
1·5
1·0
0·5
8
16
24
32
40
48
TIME (HRS.)

FIGURE 34.


HORSE 8
ACETYLPROMAZINE(ORAL-1mg/kg)
RATE OF EXCRETION(mg/hr)
1·5
1·0
0·5
8
16
24
32
40
48
TIME (HRS.)

They were detected up to 40 hours after administration and accounted for approximately 2% of the dose. Sulphate and glucuronide conjugate metabolites, on the other hand, were found only during the first 32 hours after dosing, representing 1.1% and 0.8% of the dose respectively.

Table 8 shows the percentage of the dose of acepromazine excreted as the different metabolic groups in individual experiments. Of interest are the low percentage total excretion (~4%), the presence of sulphate conjugated metabolites after this route of administration (~1%), and the predominance of unconjugated metabolites (~2%). The average ratio of unconjugated to conjugated metabolites was approximately 1:1.

(c) Metabolism of Acepromazine by the Dog.

Since an extensive literature survey revealed no previous reports on the metabolism and excretion of acepromazine or propionylpromazine, it was decided to investigate whether the routes of metabolism found for such ketonic derivatives are peculiar to the horse or are found in other species. Thus a series of experiments was undertaken to study the metabolism and excretion of acepromazine by the dog.

Acepromazine maleate was administered either orally (0.5 mg/Kg given in tablet form), or intramuscularly (1 mg/Kg as a 1% solution) to a series of 6 dogs, comprising 3 greyhounds and 3 mongrel breeds. Dogs were housed in a metabolism cage to allow collection of urine over 24 hours. 500 ml aliquots from each 24 hour sample were analysed for the six metabolic groups as described for the horse, (Pg.10.

Table 8 The Percentage Urinary Excretion of Dose after Administration of Acepromazine Maleate to the Horse.

Oral Dose (1 mg/Kg)

	Total Excreted	Glucuronides	Sulphates	Unconjugated	Ratio*
Horse 8	3.0%	0.6%	0.9%	1.5%	5:3:2
Horse 5a	5.0%	0.9%	1.2%	2.9%	10:4:3
Horse 5b	4.2%	0.9%	1.3%	2.0%	7:4:3

Average excreted = 4%
Average Ratio = 8:4:3

I.M. Dose (0.5 mg/Kg)

	Total Excreted	Glucuronides	Sulphates	Unconjugated	Ratio*
Horse 4	1.3%	Trace	Trace	1.3%	-
Horse 5	1.3%	Trace	Trace	1.3%	-
Horse 8	1.3%	Trace	Trace	1.3%	-

Average excreted = 1.3%

The ratio indicated represents Unconjugated:Sulphate conjugate:Glucuronide conjugated metabolites

TABLE 8.

It was found that metabolites were excreted in small concentration after either route of administration. Unconjugated metabolites were detected as sulphoxide derivatives of promazine, and both conjugated fractions produced ultra violet spectra with maxima varying from sample to sample as experienced with the horse. Thus metabolism of acepromazine appears to be similar for both the horse and the dog.

(d) Summary.

(i) Acepromazine and propionylpromazine undergo two major routes of metabolism. They either lose the side chain ketone group at the 2-position and are not further conjugated or retain this group and are conjugated with glucuronic or sulphuric acid. Sulphoxide formation takes place in the unconjugated fraction which predominates.

(ii) After intramuscular administration of acepromazine, both types of total excretion pattern obtained with promazine were noticed, whereas only the first type was evident after the oral route. In both cases the fall off in excretion was much more regular than for the other compounds investigated, and no metabolites were detected after 40 hours.

(iii) Unconjugated derivatives accounted for almost all metabolites detected after intramuscular administration 1.3% of dose), and approximately half the amount excreted after the oral route, when total recovery was approximately 4% of the dose.

4. Comparison of Metabolism of the Different Drugs.

(a) Conjugation of Metabolites.

The metabolic routes followed by each drug were found to depend

on the substituents on the nucleus. Thus promazine and chlorpromazine, and presumably other derivatives in which there is no ketone grouping in conjugation with the aromatic ring, are excreted predominantly as glucuronide conjugates. When promazine or chlorpromazine was administered intramuscularly the average ratio of glucuronides to unconjugated metabolites was approximately 5:1, and after oral administration the corresponding ratio was approximately 10:1. Acepromazine and propionylpromazine on the other hand, are metabolised and excreted predominantly in the unconjugated form as sulphoxide derivatives of promazine. After intramuscular administration of acepromazine the conjugated fractions were found in only trace amounts, and after oral dosing the ratio of glucuronides to sulphates to unconjugated metabolites was 3:4:8.

(b) Percentage of dose excreted.

The percentage of dose excreted also varied from compound to compound, metabolites of chlorpromazine being detected in greatest amounts. After oral administration of this drug approximately 27% of the dose was excreted, compared to 10% for promazine and 4% for acepromazine, and after intramuscular dosing the corresponding figures were 10%, 11% and 1.3%. This order for the percentage of each drug excreted, (i.e. chlorpromazine > promazine > acepromazine), cannot be related to the size of dose administered, since this decreases in the order promazine > chlorpromazine > acepromazine, the ratio being approximately 10:5:1. The percentage also takes into account differences in the size of dose.

(c) Duration of Excretion.

The duration of excretion of the different drugs was found to decrease in the order promazine > chlorpromazine > acepromazine, and this may possibly be related to dose. The determination of lengths of excretion is governed by the limits of detection of the procedure used. Using the more sensitive methods of thin layer chromatography it has been shown that metabolites can be detected for approximately 24 hours longer than by ultra violet analysis, (pg. 198), and length of excretion again appears to be related to dose.

After intramuscular administration of promazine, excretion lasted at least 96 hours in every experiment, whereas metabolites of chlorpromazine were only detected for this length of time on one occasion, and no evidence of acepromazine derivatives was found after 32 hours. After oral administration, excretion of promazine and chlorpromazine lasted between 72 and 96 hours and acepromazine metabolites were detected up to 40 hours.

(d) Total Excretion Rates.

Total rates of excretion of acepromazine metabolites with one exception, (Horse 5 after intramuscular administration), attained a maximum within 8 hours, and thereafter fell off regularly as would normally be expected. After administration of chlorpromazine this type of excretion was only noted on one occasion, (Horse 5 after oral administration), and the fall off was much more irregular. Instead, the rate tended to rise slowly and irregularly to a maximum between 16 and 40 hours, diminishing in a similar fashion. Two distinct types of excretion were noted for promazine, the first

corresponding to that found for acepromazine, but with a much more irregular fall, and the second rising slowly to a maximum between 16 and 32 hours then decreasing irregularly.

5. Effects of route of administration on excretion.

The presence of measurable quantities of sulphate conjugated metabolites after oral administration appears to be the most important difference between this route and intramuscular injection. Following oral administration of promazine, sulphate conjugated metabolites represented approximately 1.4% of the dose, and corresponding percentages for chlorpromazine and acepromazine were 1.3% and 1.1%. After intramuscular administration, in every case, sulphate conjugated metabolites were found in only trace amounts. This fraction accounted for 1.2% of the dose after intravenous administration of promazine. This will be discussed later (Pg. 269).

A further difference between oral and intramuscular administration was the much larger proportion of the dose excreted after the oral route. This was most noticeable for chlorpromazine or acepromazine. For chlorpromazine there was a clear difference, the total percentages excreted being 27% after oral administration and 10% by the intramuscular route. Corresponding percentages for acepromazine were 4% and 1.3%. With promazine, however, the average percentages of the dose excreted were approximately the same for both routes, being 10% after oral, and 11% after intramuscular administration.

Figures 35, 36 and 37 show the percentages of dose excreted over successive 8 hour intervals after different routes of administration

of promazine, chlorpromazine, or acepromazine. For all three drugs metabolites were excreted in greater amounts after oral administration during the first 32 hours than after intramuscular injection. Thereafter the rate was approximately the same for both routes. After intravenous administration of promazine rates of excretion were of the same order over the first 32 hours as for oral administration.

Other differences noted were in the lengths of excretion after the different routes. After intramuscular administration of promazine, metabolites were detected for at least 96 hours in every experiment, whereas after oral dosing excretion was complete by 64 hours. The corresponding length of excretion after intravenous administration, determined in a single experiment, was 48 hours. For chlorpromazine, excretion lasted between 80 and 96 hours after oral, and, with one exception, for 64 hours after intramuscular administration. The length of excretion of metabolites of acepromazine was approximately the same for both routes of administration, being 40 hours after oral dosing and 32 hours after intramuscular injection.

6. Comparison of Individual Horses.

The amounts and types of metabolites excreted during these experiments could neither be related to the age nor the sex of the horses. However peculiarities were occasionally noted for individual animals.

(a) Excretion by Horse 4.

Horse 4 excreted metabolites in much greater amounts than

FIGURE 35.

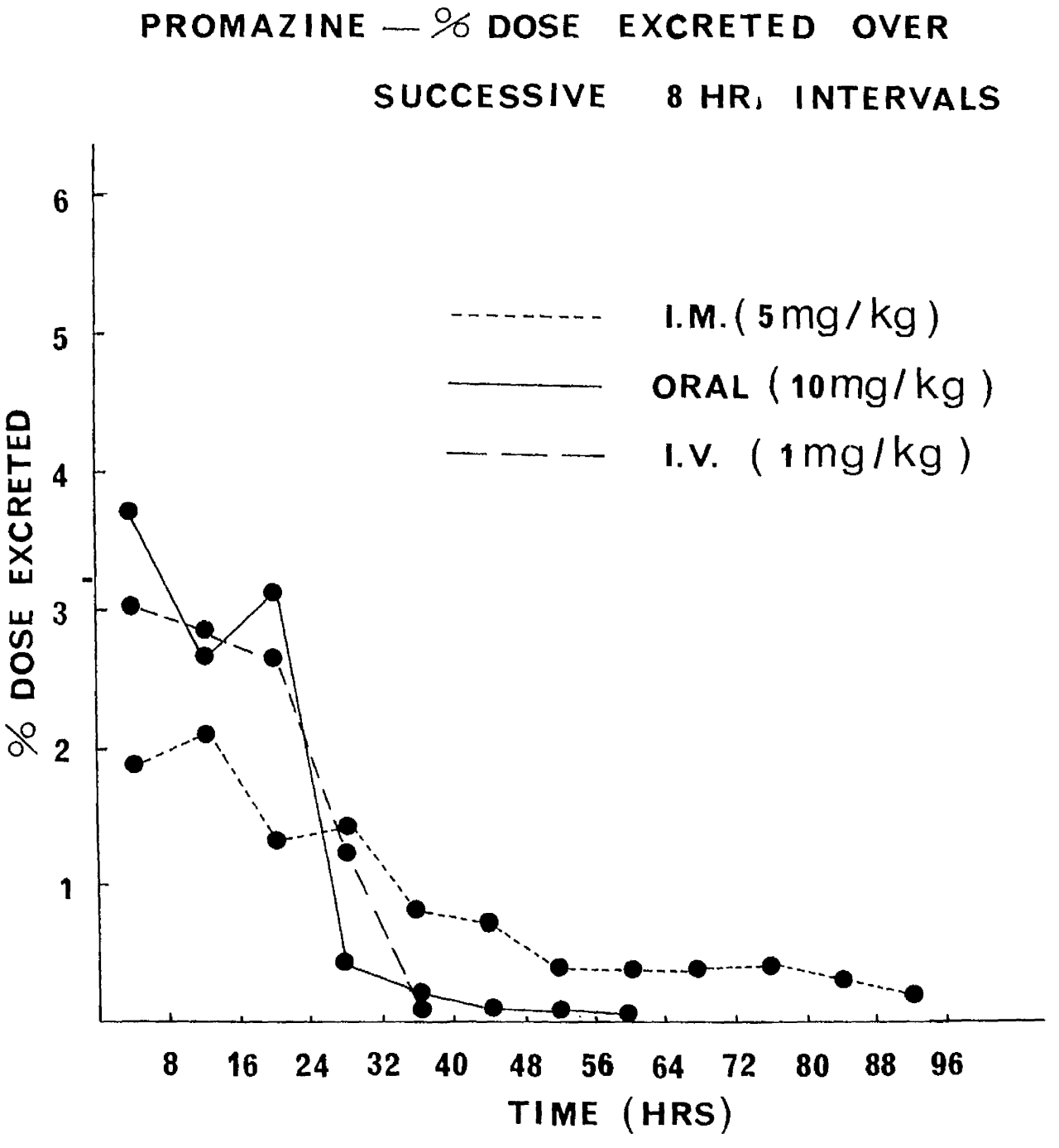

PROMAZINE — % DOSE EXCRETED OVER
SUCCESSIVE 8 HR. INTERVALS
I.M. (5mg/kg)
ORAL (10mg/kg)
I.V. (1mg/kg)
% DOSE EXCRETED
6
5
4
3
2
1
8
16
24
32
40
48
56
64
72
80
88
96
TIME (HRS)

FIGURE 36.

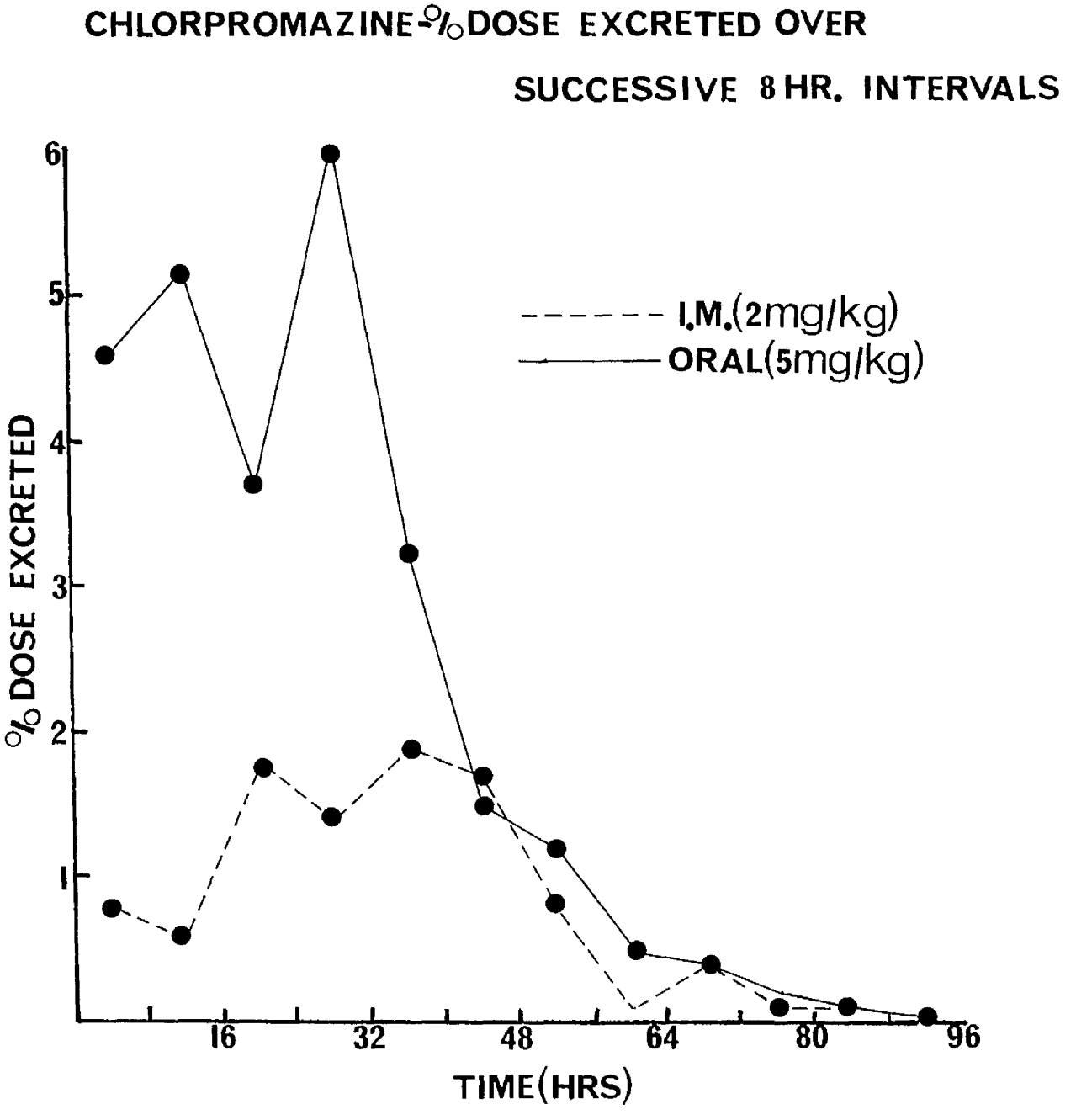
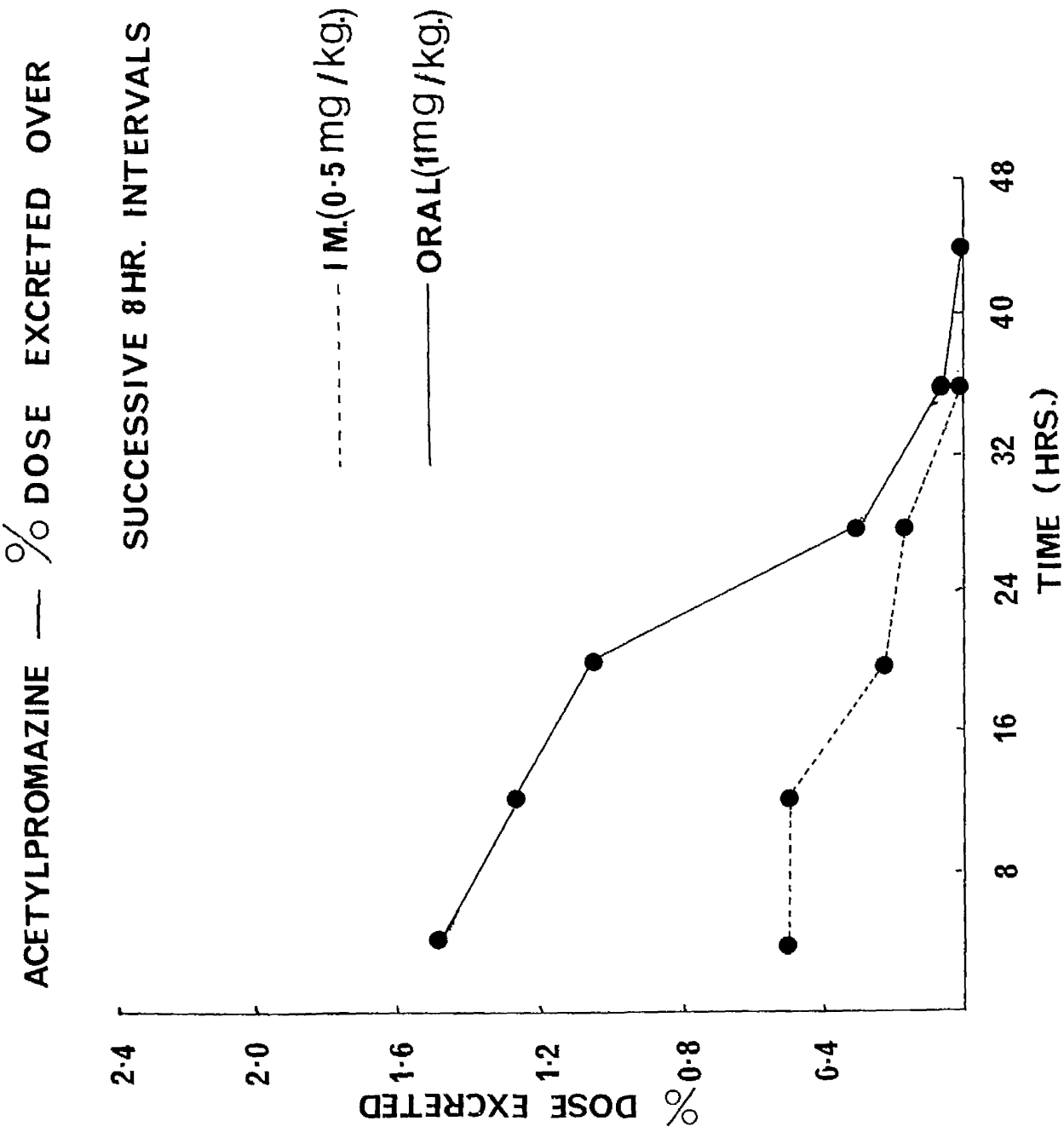

CHLORPROMAZINE-%DOSE EXCRETED OVER
SUCCESSIVE 8 HR. INTERVALS
I.M.(2mg/kg)
ORAL(5mg/kg)
% DOSE EXCRETED
6
5
4
3
2
1
16
32
48
64
80
96
TIME(HRS)

FIGURE 37.


ACETYLPROMAZINE — % DOSE EXCRETED OVER SUCCESSIVE 8HR. INTERVALS
I M.(0·5 mg / kg.)
ORAL(1mg / kg.)
% DOSE EXCRETED
2·4
2·0
1·6
1·2
0·8
0·4
8
16
24
32
40
48
TIME (HRS.)

other horses. After intramuscular administration of promazine urinary metabolites accounted for approximately 22% of the dose which was more than twice the percentage excreted by other horses. Similarly, after oral administration of promazine it excreted 13.6% of the dose, whereas horses 5 and 8 excreted 9.3% and 7.4% respectively. Corresponding percentages after intramuscular administration of chlorpromazine were 18.5% for horse 4 compared to 5.5% and 4.6% for horse 5.

The larger percentages appear to be due to a larger excretion of the glucuronide conjugated fractions by this horse. After intramuscular administration of promazine it excreted 18.1% of the dose in this form compared to an average of 7.4% for the other five experiments (9.6%, 7.8%,7.5%, 6.1% and 5,9%). The corresponding figures after intramuscular chlorpromazine were 17.2% compared to 4.8% and 3%, and after oral administration of promazine were 11.2% compared to 5.2% and 6%. Percentages excreted in the unconjugated form ware of the same order within each series of experiments, as was the case for the sulphate fraction.

After intramuscular administration of chlorpromazine, horse 4 excreted metabolites for at least 96 hours whereas no metabolites were detected from the other two horses after 64 hours. However after both routes of administration of promazine the length of excretio by all horses (including horse 4) was of the same order within each series of experiments (96 hours after intramuscular and 64 hours after oral administration). Thus the larger amounts of metabolites excreted by horse 4 must be due to a greater rate of metabolism and

not to prolonged excretion of metabolites.

Unconjugated metabolites of promazine and chlorpromazine were excreted by most horses entirely as sulphoxide derivatives. Horse 4 however once more proved an exception. After intramuscular administration of promazine it also excreted this fraction in the sulphide form from 32 hours after injection until the end of the experiment, and after oral administration such derivatives were detected during the first 24 hours. However, the larger percentages of metabolites excreted by this horse were not a reflection on the presence of these sulphide derivatives. Unconjugated sulphide metabolites were also excreted in trace amounts by Horse 7 from 48 to 72 hours after oral administration of chlorpromazine.

(b) Excretion of conjugated metabolites.

Glucuronide conjugated metabolites of promazine or chlorpromazine, as a rule, were excreted almost entirely as sulphide derivatives. Horse 5,however, after intramuscular administration of chlorpromazine excreted this fraction predominantly in the sulphoxide form, only trace amounts of sulphide derivatives being detected. It also excreted glucuronide conjugated sulphoxide metabolites after oral administration of promazine or chlorpromazine, and after intramuscular administration of promazine.

Similarly, although sulphate conjugated metabolites of promazine or chlorpromazine were normally excreted almost entirely in the sulphide form, small amounts of sulphoxide derivatives were occasionally detected. They were excreted by horses 5 and 7 between 40 and 64 hours after oral administration of chlorpromazine

and were also found towards the end of excretion of the sulphate fraction after oral administration of promazine to horse 8 (56 to 72 hours after dosing.)

(c) Consistency of excretion patterns for individual horses.

The consistency of patterns of metabolism and urinary excretion for individual horses was investigated on three occasions by repetitio of experiments. Comparison of results from such duplicate experiments showed that, for the most part, metabolism and excretion proceeded along similar lines.

Horse 5 received promazine intramuscularly on two occasions. The respective percentages of dose excreted were similar, (9.1% and 7.1%), but the ratio of the glucuronide fraction to the other two fractions combined was considerably different. On the first occasion this was ~2:1, whereas on the second occasion, the figure was ~6:1. In both experiments unconjugated metabolites were excreted as sulphoxides up to 96 hours. However traces of sulphide derivatives, absent in the first experiment, were detected during the first 40 hours on the second occasion. In both cases glucuronide conjugated sulphoxides were excreted over 96 hours although traces of sulphide derivatives were also found during the first 32 hours of the second experiment. Sulphate conjugated metabolites were found in only trace amounts on both occasions.

This horse also received chlorpromazine intramuscularly on two occasions. Again the total percentages excreted (5.5% and 4.6%) were similar, whereas some difference existed in the ratio of glucuronides to the other fractions (7:1 and 2:1). In both cases

unconjugated metabolites were excreted entirely in sulphoxide form although, in the first experiment, samples collected during the first 16 hours contained no trace of this fraction. Similarly glucuronide conjugated metabolites were excreted on both occasions as sulphoxides up to 56 hours after injection. The corresponding sulphide derivatives were found in only trace amounts. As already described, excretion of glucuronide conjugates in the sulphoxide form seems to be a peculiarity of this horse (Pg. 167).

Horse 7 received chlorpromazine orally on two occasions. In this case the percentages of the dose excreted differed considerably, being 39.5% and 15.6%. However, the ratio of glucuronide to unconjugated metabolites was similar (~ 22:1 and 16:1). In both cases unconjugated metabolites were excreted in the sulphoxide form for approximately 72 hours, although traces of unconjugated sulphides were found during the first experiment between 48 and 72 hours of dosing. Glucuronide conjugated metabolites were excreted entirely as sulphides over 80 hours on both occasions. Sulphates were excreted predominantly as sulphide derivatives for 56 hours on the first occasion and for 72 hours in the second experiment. However, in both cases trace amounts of sulphoxides were also detected when excretion of this fraction was almost complete. These results are summarised in Table 9.

7. Initiation and Termination of Excretion.

In addition to pooling urine samples over 8 hour periods, 100 ml aliquots from the first sample voided after drug administration were analysed in an attempt to determine how soon metabolites were

TABLE 9.

Experiment	Horse	Drug	Route	% Excreted	Ratio*	Unconjugated	Glucuronides	Sulphates
1	5	Promazine	I.M.	9.1	2:1	SO	SO	Trace
2				7.1	6:1	SO	SO	Trace
1	5	Chlorpromazine	I.M.	5.5	7:1	SO	SO	Trace
2				4.6	2:1	SO	SO	Trace
1	7	Chlorpromazine	Oral	39.5	18:1	SO	S	S[redacted]
2				15.6	8:1	SO	S	S[redacted]

* Denotes ratio of glucuronides to other two fractions. SO = Sulphoxides. S = Sulphides.

Table 9. Comparison of Duplicate Experiments on Individual Horses.

excreted. Also, in experiments where excretion had previously been found to last for at least 96 hours, collection was continued up till 102 hours and thereafter at 24 hour intervals until metabolites were no longer detected.

Such experiments have shown that, for the most part, excretion of metabolites does not commence until between 2 and 4 hours after administration. After intramuscular administration of chlorpromazine to horse 5, no metabolites were found in the first two pooled eight hour samples, whereas on the second occasion they were detected within 3 hours in the first sample voided. Initiation of excretion for the other horse (4) dosed in this manner was within 4 hours of injection.

During all three experiments after oral administration of chlorpromazine, metabolites were detected in the urine within 4 hours of dosing. The first sample obtained from horse 7 on each occasion, at 30 minutes and 1 hour respectively after administration, contained no metabolites.

After oral, intramuscular, or intravenous administration of promazine, excretion of metabolites did not commence until between 2 and 4 hours. A sample was obtained from horse 5 within 40 minutes of oral administration but it contained no metabolites. However, on both occasions that this horse received an intramuscular dose of promazine, excretion had commenced in samples collected 90 minutes after administration.

Metabolites of acepromazine, with one exception, were detected within 3 hours of administration. No urine was voided by horse 4 until 16 hours after intramuscular injection, but the first sample

had a strong concentration of metabolites. Also a sample collected from horse 5 within 90 minutes of intramuscular administration, contained measurable amounts of metabolites.

In experiments where metabolites were excreted over 96 hours, (i.e. intramuscular injection of promazine or chlorpromazine), they were still detectable at 102 hours after administration, but, in every case, excretion was complete after a further 24 hour period.

8. Urinary Volume and pH.

As previously described, both urinary volume and pH were measured for each sample passed. On average, samples were obtained every 3 hours. However, occasionally two samples were obtained within 1 hour , and, on one occasion 16 hours elapsed before the first sample was passed. Also urine was excreted much more frequently between 10 p.m. and 10 a.m., but this did not result in an increase in urinary volume over the period.

Volumes of urine collected over successive 8 hour intervals ranged between 1000 and 2500ml, and the average volume voided was approximately 1600 ml. Individual samples ranged in volume from 200 to 1500 ml. However, on several occasions volumes of less than 100 ml were obtained, and on one occasion 2500 ml was voided. There was no relation between frequency of excretion and the volume of urine passed.

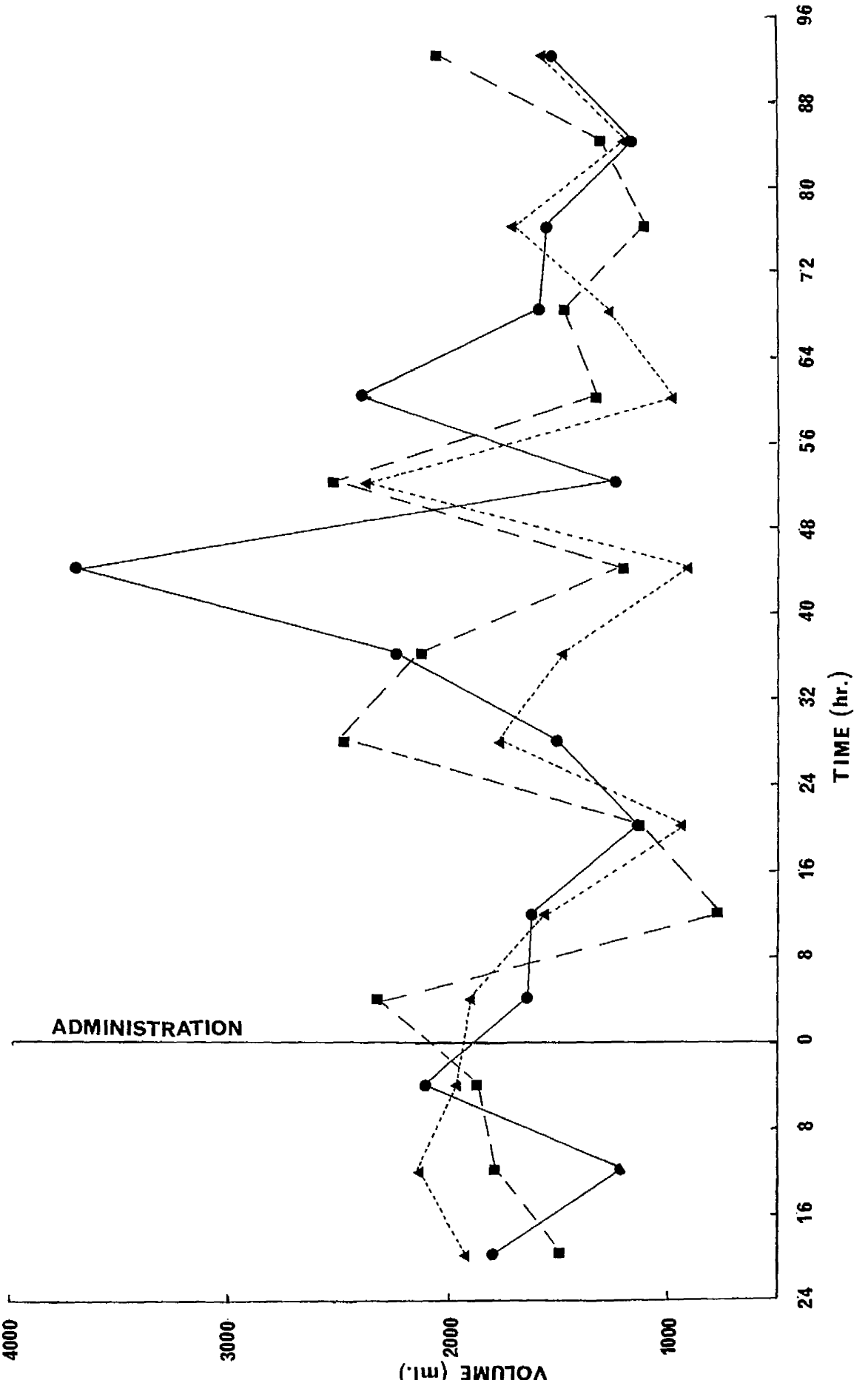
On several occasions it was noted that the frequency of excretion was much less during the first 24 hours after drug administration than for the remainder of the experiment. It was

thought that this might be due to an antidiuretic effect of such drugs on the horse. To clarify the situation urine collections were made from three horses for 24 hours before drug administration, and volumes were compared with those subsequently passed. As can be seen from Figure 38, there is a decrease in urinary volume in every case during the first 24 hours. However, this could be due to a decrease in water intake during the maximum effects of the drug.

Urinary volumes were also compared after oral and intramuscular administration of each drug to investigate any relationship between them and the greater percentages of dose excreted after oral administration. Figure 39 shows the average urinary volumes passed over successive 8 hour intervals after oral or intramuscular administration of chlorpromazine. However, no obvious difference can be seen between the volumes voided after each route. This was also true for acepromazine and promazine.

The pH of urine samples, varied between 7.0 and 8.5. However, occasionally it varied between 5 and 7 over a whole experiment, but was not related to an increase in amounts of metabolites excreted. Table 10 shows the volume, pH, and rate of excretion for successive 8 hour samples over the first 48 hours after administration of chlorpromazine. As can be seen there is no obvious relationship between the rates and the volume or pH of the samples. This was also true for promazine and acepromazine. After intramuscular administration, several samples from horse 4 had a pH of less than 7 and rates of excretion were greater than in corresponding samples

FIGURE 38.



URINARY VOLUMES BEFORE AND AFTER DRUG ADMINISTRATION.

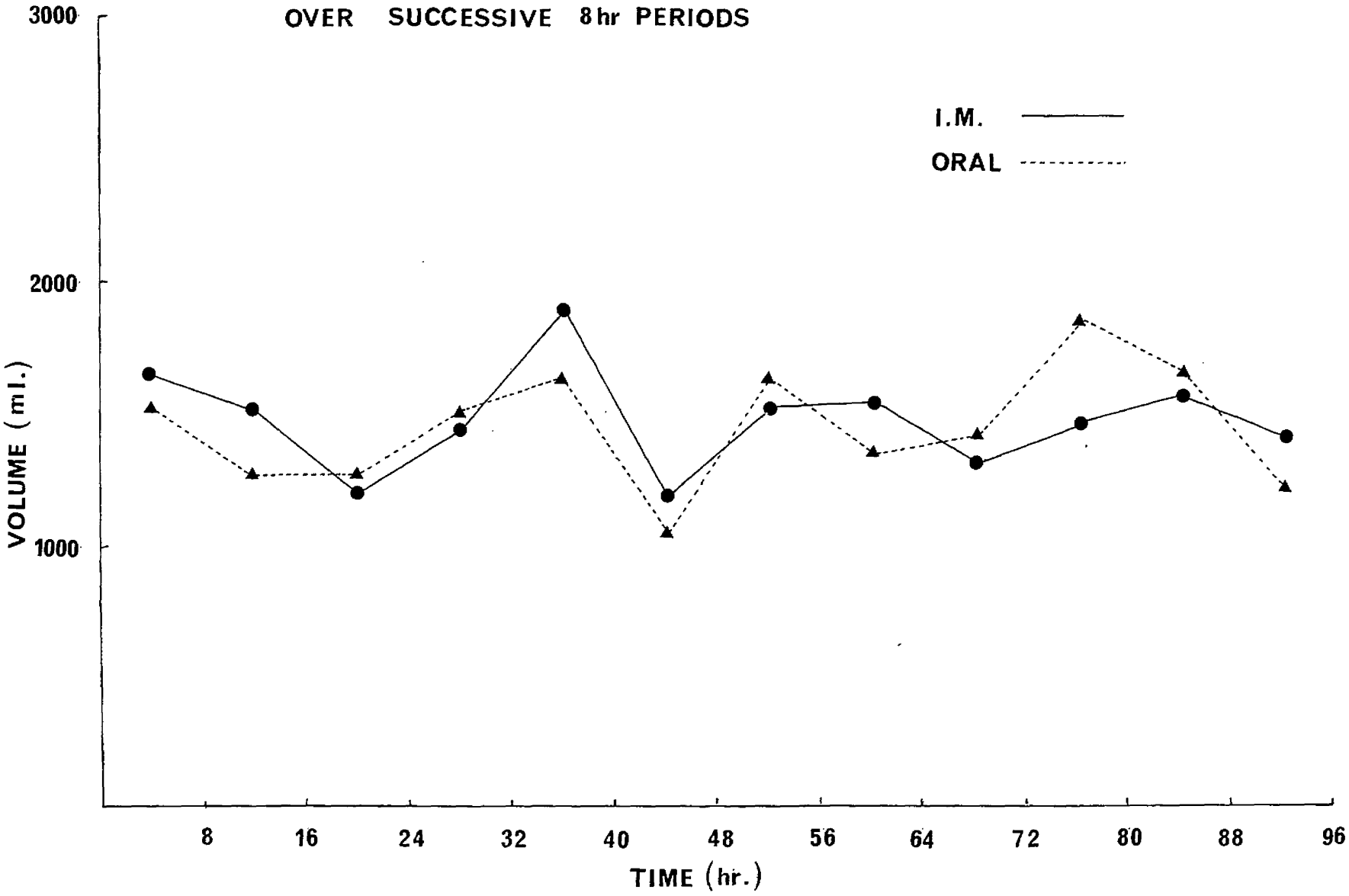

CHLORPROMAZINE. AVERAGE URINARY VOLUME
OVER SUCCESSIVE 8 hr PERIODS
I.M.
ORAL
VOLUME (ml.)
3000
2000
1000
8
16
24
32
40
48
56
64
72
80
88
96
TIME (hr.)

FIGURE 29.

Table 10 Volume, pH and rate of excretion of metabolites for successive 8 hour samples after administration of chlorpromazine hydrochloride to the horse.

Intramuscular Administration (2 mg/Kg)

	Horse 4			Horse 5a			Horse 5b		
Sample	Volume(ml)	pH	Rate(mg/hr)	Volume(ml)	pH	Rate(mg/hr)	Volume(ml)	pH	Rate(mg/hr)
I	1550	5.8	1.6	1820	7.7	1.0	1580	7.9	0.7
II	1660	7.7	0.6	1310	7.8	1.0	1620	8.0	0.8
III	1530	6.1	5.7	1160	8.1	1.3	930	7.6	0.7
IV	1860	6.1	3.9	1380	7.0	1.5	1080	7.9	0.6
V	2350	6.7	5.0	1480	7.2	1.1	1820	7.9	2.1
VI	1700	7.4	5.6	780	7.2	0.7	1100	7.7	0.8

Oral Administration (5 mg/Kg)

	Horse 5			Horse 7a			Horse 7b		
Sample	Volume(ml)	pH	Rate(mg/hr)	Volume(ml)	pH	Rate(mg/hr)	Volume(ml)	pH	Rate(mg/hr)
I	930	7.9	35.5	1910	8.0	8.4	1730	8.1	Trace
II	620	8.1	11.8	1560	7.9	31.6	1650	8.3	6.8
III	1550	7.2	4.8	930	7.7	17.4	1355	8.0	13.1
IV	1560	7.9	12.1	1710	8.0	30.1	1150	7.4	15.5
V	1730	7.5	8.2	1500	7.3	18.6	1640	7.5	3.4
VI	1380	7.9	9.1	900	7.6	2.3	1690	7.2	2.4

TABLE 10.

from horse 5. However, samples with a pH of more than 7 also produced greater rates of excretion than horse 5. As previously described (Pg.182) greater rates of excretion than normal at pH values above 7 were a noticeable feature of metabolism by Horse 4. Thus the increased rates must be attributed to some other aspect of metabolism by the horse, and not to the acidic pH of its urine.

8. Summary.

(i) Two types of excretion were noted. In the first the rate reached a maximum within 8 hours of administration, whereas the second type rose slowly to a maximum between 16 and 40 hours. Acepromazine, followed the first course, whereas both patterns were noted after administration of promazine or chlorpromazine.

(ii) Percentages of dose recovered were low, being approximately 19% for chlorpromazine, 10% for promazine, and 3% for acepromazine. Excretion of promazine and chlorpromazine was also prolonged, in certain cases lasting more than 96 hours. No metabolites of acepromazine were detected after 40 hours.

(iii) Using promazine or chlorpromazine conjugated metabolites predominated, whereas, after administration of acepromazine or propionylpromazine, the unconjugated fraction was in greatest evidence. Sulphate conjugated derivatives of each drug were present in small amounts and were only detected in trace quantities after intramuscular administration.

(iv) Unconjugated metabolites of promazine and chlorpromazine were mostly in the sulphoxide form, whereas glucuronides and sulphates

were excreted as sulphides. On the other hand, acepromazine and propionylpromazine were metabolised in the unconjugated fraction as sulphoxide derivatives of promazine and in the conjugated fractions as a mixture of sulphide and sulphoxide metabolites of the parent drug.

(v) The percentage of dose excreted decreased in the order chlorpromazine > promazine > acepromazine, whereas the duration of excretion fell off in the order promazine > chlorpromazine > acepromazine.

(vi) Larger percentages were recovered after oral administration than after intramuscular injection.

(vii) No relation was found between amounts or types of metabolites excreted and the age or sex of the animals. In duplicate experiments on individual horses metabolism and excretion proceeded along similar lines although several differences were noted.

(viii) Excretion of metabolites commenced between 2 and 4 hours after administration, and when excretion lasted longer than 96 hours no metabolites were detected by 126 hours. No correlation was found between urinary volume, pH and amounts of metabolites excreted.

C. Qualitative Studies of Metabolism.

Excretion of metabolites was also followed over successive 8 hour intervals using thin layer chromatography. This technique

provided separation of individual metabolites for subsequent structur determination. 300 ml aliquots from each 8 hour sample were extracted for chromatography as previous'y described (Pg.113),and spotted on consecutive 1 cm. strips of a chromatography plate. Development and subsequent colour formation gave a qualitative picture of excretion of individual metabolites. The residual urine was pooled, and used for further structural determination.

(1) Metabolism of Promazine.

(a) Unconjugated Metabolites.

Figure 40 shows a typical chromatogram of unconjugated metabolites extracted after intramuscular administration of promazine hydrochloride. Marked variations exist in the concentration and number of metabolites detected in successive samples. In some cases even larger variations in intensity were noted, occasionally to such an extent that a metabolite excreted during the initial stages of an experiment was not detected in a few subsequent samples, but later reappeared. This was specially true of metabolites P1 and P3, and was noticed once for P2. In addition, trace amounts of metabolites other than those recorded were occasionally detected. The most commonly occurring had Rf values of 0.73 (Q1) (i.e. between M1 and P5) and 0.23 (Q2) (between P1 and P2) in the methanol/acetic acid/water system,and gave a reddish pink colour with sulphuric acid.

At least seven unconjugated metabolites were detected by their colour reaction with 50% sulphuric acid, and their fluorescence under ultra violet light. Further investigation of their structure was carried out by comparison with known standards in three

FIGURE 40.

UNCONJUGATED METABOLITES OF PROMAZINE

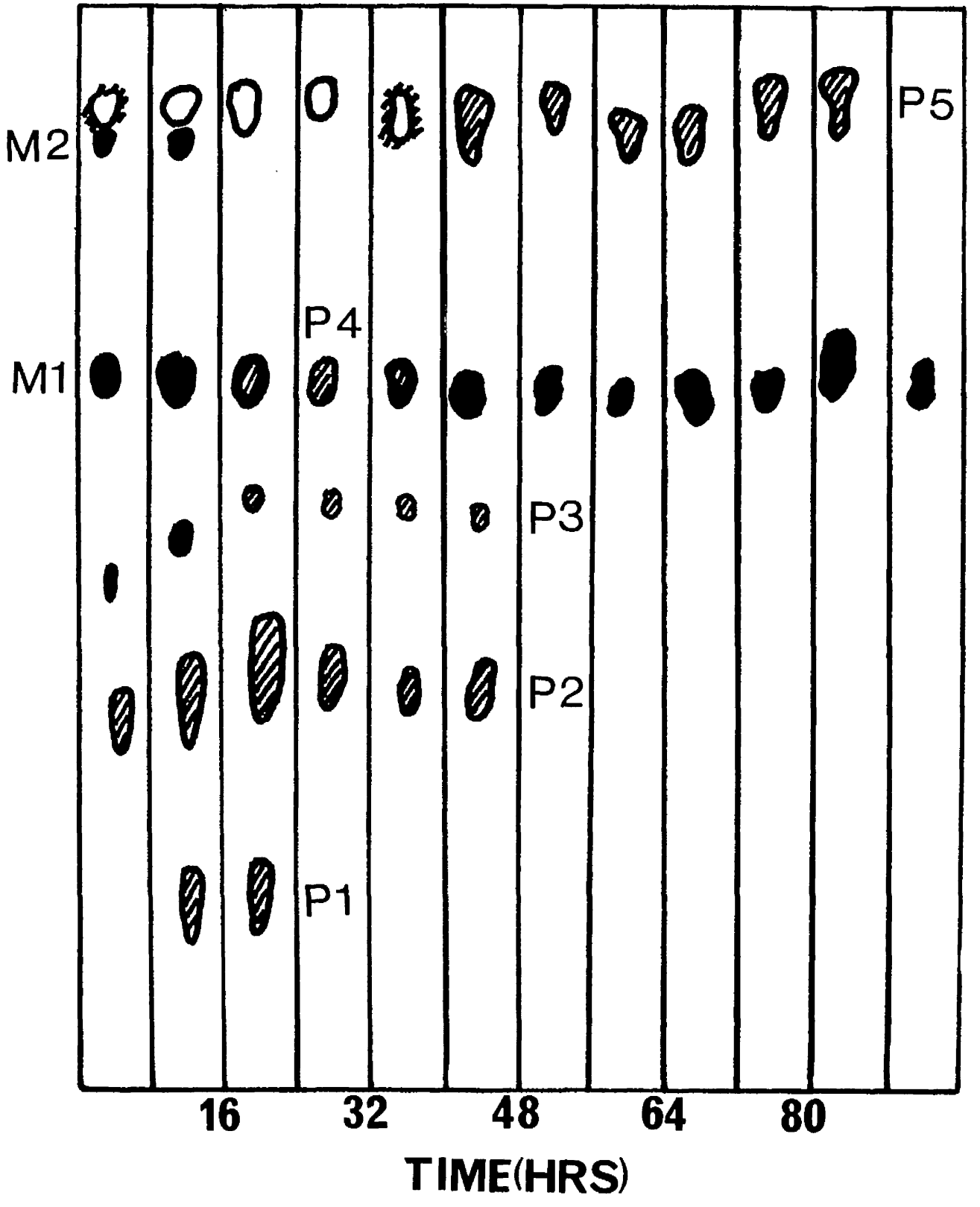


Table 11 Chromatographic, chemical and spectral properties of the unconjugated metabolites of promazine.

	Rf values			Colour Reactions					
Metabolite	System 1	System 2	System 3	H_2SO_4*	Ferric Chloride	Periodate	Ninhydrin	Nitroprusside	U.V. Max.
P1	0.17	0.01	0.15	A	-	-	-	-	270
P2	0.39	0.03	0.61	A	-	-	-	-	270
Promazine sulphoxide	0.40	0.04	0.61	A	-	-	-	-	270
P3	0.54	0.09	0.44	A	-	-	-	-	270
P4	0.65	0.10	0.95	A	+	-	-	-	250
Promazine	0.65	0.10	0.96	A	+	-	-	-	250
P5	0.92	0.12	0.57	A	-	-	-	-	270
M1	0.63	0.14	0.93	B	+	+	-	-	250
3-hydroxypromazine	0.61	0.15	0.93	B	+	+	-	-	250
M2	0.88	0.18	0.98	B	+	+	-	-	250

TABLE 11.

* A denotes pink colour reaction
* B denotes purple colour reaction.

System 1 = Methanol/Acetic Acid/Water (5:3:2)
System 2 = Ethanol/Acetic Acid (1:1)
System 3 = Chloroform/Acetone/Diethylamine (2:7:1)

solvent systems, the use of specific spray reagents, and elution techniques (Pg.116). A summary of the results is shown in Table 11.

Of the seven metabolites detected, three have been positively identified. P2 had the same Rf values as promazine sulphoxide, (0.39, 0.03 and 0.61), in each solvent system and, on elution with 0.1N hydrochloric acid, its ultra violet spectrum corresponded to that of a sulphoxide derivative of promazine. It had a pink colour reaction with 50% sulphuric acid, and no reaction was observed using the periodate spray, confirming the absence of a hydroxyl function. No colour was observed with the ferric chloride solution, confirming the oxidation of the ring sulphur to the sulphoxide form, and tests for demethylation of the side chain using ninhydrin or nitroprusside sprays were also negative. It was thus concluded that this metabolite was promazine sulphoxide.

Spot P4 was identified as the parent drug. Both compounds ran to the same heights in the systems used and, on elution, this derivative had an ultra violet spectrum identical to that of promazine. A reddish pink reaction with sulphuric acid, and no reaction with sodium periodate, indicated that hydroxylation had not occurred. A reddish pink reaction to the ferric chloride spray confirmed that the ring sulphur atom was unoxidised, and tests for demethylation using ninhydrin or nitroprusside both proved negative.

The Rf values for M1, (0.63, 0.14, and 0.93) were almost identical to those of 3-hydroxy promazine, (0.61, 0.15, 0.93), and its ultra violet spectrum correspond to that of a sulphide derivative of promazine. The presence of a hydroxyl function was

confirmed with both 50% sulphuric acid and sodium metaperiodate. There was no evidence of demethylation using either of the specific amine spray reagents, and a reddish pink coloration with the ferric chloride spray confirmed that the compound was unoxidised at the sulphur atom. Thus M1 was identified as 3-hydroxy promazine.

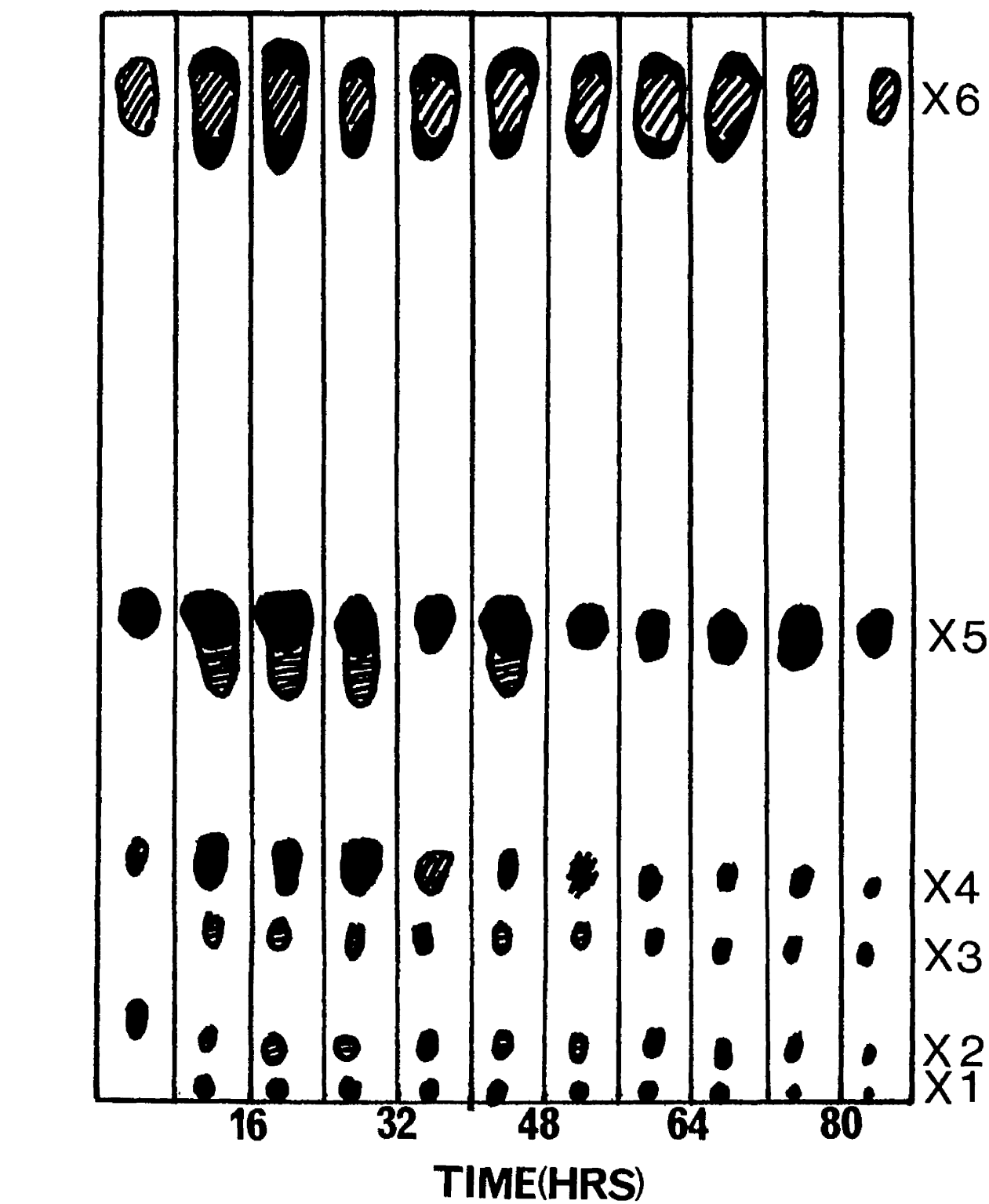
Due to a lack of reference standards the other four metabolites were not completely identified. Spots P1, P3 and P5 produced a pink coloration with sulphuric acid, and their ultra violet spectra were typical of sulphoxide derivatives. This was confirmed by a lack of reaction with the ferric chloride spray. There was no evidence of demethylation or hydroxylation. Spot M2, on the other hand, had a purple reaction with both sulphuric acid and periodate indicating that it was hydroxylated. It had a reddish pink reaction with the ferric chloride spray, and its ultra violet spectrum was typical of a sulphide derivative. There was no evidence of demethylation.

Thus the unconjugated urinary metabolites of promazine in the horse are promazine sulphoxide (P2), 3-hydroxpromazine (M1), the parent drug (P4), three sulphoxide derivatives which do not appear to be demethylated or hydroxylated (P1, P3, P5), and a hydroxylated derivative occurring in the sulphide form (M2).

(b) Glucuronide Conjugated Metabolites.

Figure 41 shows a typical chromatogram of glucuronide conjugated metabolites of promazine excreted over successive 8 hour intervals after intramuscular administration. The

FIGURE 41.


PROMAZINE-GLUCURONIDE CONJUGATES
X6
X5
X4
X3
X2
X1
16
32
48
64
80
TIME(HRS)

metabolites shown are not the actual conjugated forms, but their genins obtained after hydrolysis with β-glucuronidase. As with the unconjugated metabolites, excretion was still detectable at 96 hours, although variations in intensity and numbers of spots from sample to sample were not so marked.

At least 11 metabolites were detected using the sulphuric acid spray and fluorescence under ultra violet light, six of which,(X1 - X6), constituted the major fraction. Spots X1 - X5 reacted both with sulphuric acid and periodate indicating that hydroxylation had occurred. X6,on the other hand, showed no evidence of hydroxylation. None of the metabolites showed any reaction with ninhydrin or nitroprusside sprays. Each spot was eluted with 0.1N hydrochloric acid and the ultra violet spectra of the eluates were recorded. X1, X2, X4 and X5 had spectra typical of sulphide derivatives of promazine, whereas those of X3 and X6 corresponded to the sulphoxide form. Their reaction, or lack of reaction, with ferric chloride solution confirmed these states of oxidation.

X5, the major metabolite, had similar Rf values, (0.40, 0.53, 0.60), in each solvent system to 3-hydroxy-promazine, (0.41, 0.55, 0.61), and, as previously described, behaved chemically as a hydroxylated sulphide derivative. Further confirmation of the position of hydroxylation was obtained by elution of X5 with methanol, and subsequent reaction with 50% sulphuric acid as described by Beckett, Curry and Salt, (1964). The combined ultra violet and visible spectrum of the product had maxima at 278, 343, 372 and 556 mμ differentiating it from the 2- and 4- derivatives by the peak at

Table 12. Chromatographic, Chemical and Spectral Properties of the Glucuronide Conjugated Metabolites of Promazine.

Metabolite	Rf values			Colour Reaction					U.V. Maximum.
	System 1	System 2	System 3	H_2SO_4*	Ferric Chloride	Periodate	Ninhydrin	Nitroprusside	
XI	0.01	0.03	0.05	B	+	+	-	-	250
X2	0.08	0.06	0.15	B	+	+	-	-	250
X3	0.14	0.23	0.30	B	-	+	-	-	270
X4	0.24	0.35	0.49	B	+	+	-	-	250
X5	0.40	0.53	0.60	B	+	+	-	-	250
3-hydroxypromazine	0.41	0.55	0.61	B	+	+	-	-	250
X6	0.90	0.95	0.89	A	-	-	-	-	270

TABLE 12.

* A denotes pink colour reaction
B denotes purple colour reaction

System 1 = Acetone/Isopropanol 1% Ammonia (9:7:4)
System 2 = Ethyl Acetate/Methanol/Diethylamine (7:2:1)
System 3 = Methanol/Acetic Acid/Water (5:3:2)

372 mμ. The reaction was also attempted with the other five metabolites but it was impossible to isolate enough of each to obtain a satisfactory spectrum.

Thus, after administration of promazine hydrochloride to the horse, at least 11 metabolites are excreted conjugated with glucuronic acid. Six of these, X1 - X6, were detected in every sample analysed. X5 was identified as 3-hydroxy promazine and corresponds to spot 11 in the unconjugated fraction. Spots X1 - X4 were also hydroxylated but due to a lack of reference standards, and difficulty in elution, the exact position of hydroxylation in each metabolite could not be determined. X1, X2 and X4 are sulphide derivatives, whereas X3 is in the sulphoxide form, and none of them appeared to be demethylated. The remaining metabolite, (X6), shows no evidence of demethylation, and is in the sulphoxide form. Conjugation could take place through a side chain hydroxyl group which would have no reaction with the periodate spray. The other metabolites only appeared occasionally and tended to give a blue colouration with sulphuric acid compared to the purple spots of the hydroxylated derivatives already recorded. The chemical, spectral and chromatographic properties of the glucuronide metabolites are shown in Table 12.

(c) Sulphate conjugated metabolites.

Sulphate conjugated metabolites were detected in only small amounts after intramuscular administration, during the first 48 hours. Four metabolites were detected which corresponded in physical and chemical properties to the drugs X2, X3, X5 and X6.

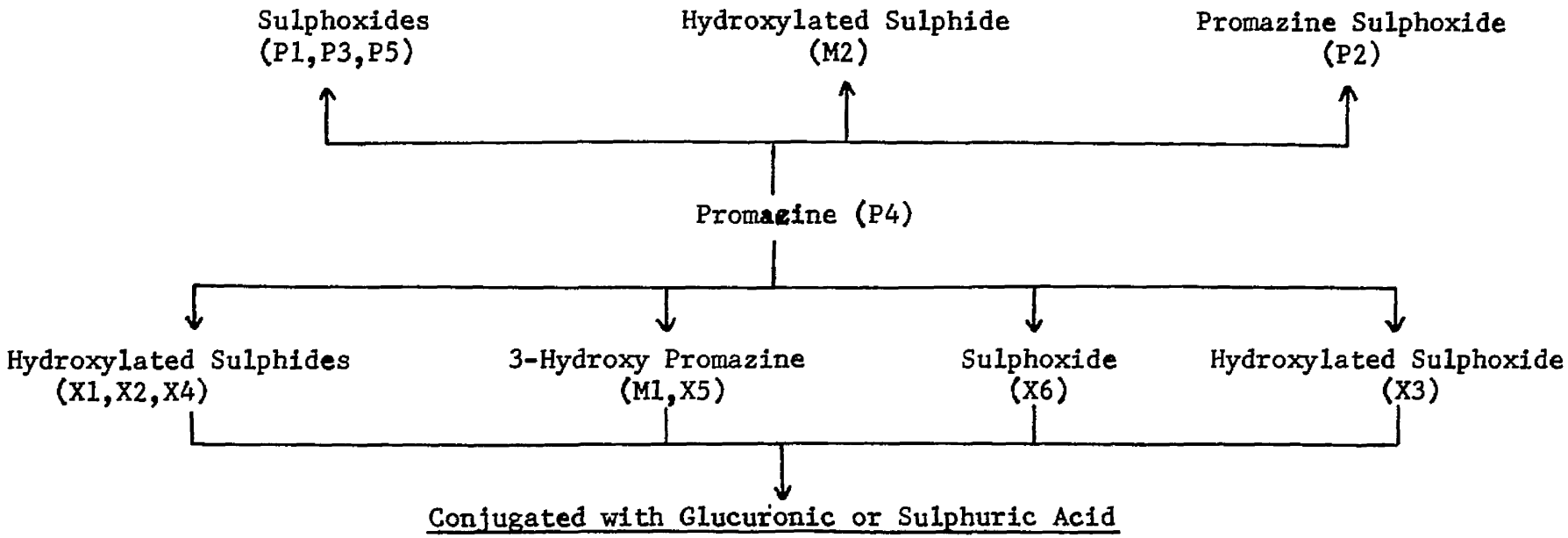


FIGURE 42.

Figure 42 Schematic diagram of metabolism of promazine by the horse

obtained after hydrolysis of the glucuronide conjugates. From the size and intensity of the spots they appeared to be present in equal concentration.

(d) Oral Administration.

Similar metabolic patterns were obtained in each fraction after oral administration. Metabolites P1 - P5, M1 and M2 were again detected in the unconjugated fraction, and spots X1 - X6 were detected in the glucuronide fraction, but in both cases they were only found up to 80 hours after administration. The sulphate conjugated group were found in much greater amounts than after intramuscular administration and were also detected up to 80 hours. In this case five sulphate conjugates were found which, from their chemical, spectral, and chromatographic behaviour, were identified as compounds X2 - X6. Further experiments using continuous extraction of 2 litre volumes of urine showed that compound X1 is also present conjugated with sulphuric acid.

The results for both routes of administration suggest that the main pathway of metabolism of promazine in the horse is hydroxylation followed by conjugation, predominantly with glucuronic acid, and to a lesser extent with sulphuric acid. A summary of the main metabolic products of promazine in the horse is given in Figure 42

(2) Metabolism of Chlorpromazine.

(a) Unconjugated metabolites.

Marked variations in the intensity and number of spots were again noted from sample to sample, and certain metabolites were

found to disappear for a number of samples, only to reappear later. Figure 43 shows a typical chromatogram of unconjugated metabolites excreted over successive 8 hour intervals after intramuscular administration of chlorpromazine hydrochloride. In addition to the derivatives recorded trace amounts of unconjugated metabolites were found with Rf values of 0.83, (CQ1), (between metabolite H1 and the urinary pigments), and 0.50, (CQ2),(attached to the top of metabolite CP3). The former gave a reddish pink reaction with sulphuric acid, and the latter a purple colour. No reaction was observed with any of the other spray reagents and, due to the small amounts excreted, no interpretable ultra violet spectra were obtained on elution.

Five unconjugated metabolites were detected by their colour reaction with sulphuric acid, and their fluorescence under ultra violet light. Their chemical, spectral and chromatographic properties are summarised in Table 13. Four, (CP1-4), had a reddish pink reaction with 50% sulphuric acid. The fifth (H1) had a purple reaction with both this reagent and sodium metaperiodate indicating that hydroxylation had taken place.

Spot CP3 ran to the same height as chlorpromazine sulphoxide in each solvent system, and their ultra violet spectra were identical. No reaction was observed with ninhydrin, nitroprusside, periodate or ferric chloride confirming that the compound is a sulphoxide derivative in which neither hydroxylation nor demethylation has taken place. Thus spot CP3 was identified as chlorpromazine sulphoxide.

FIGURE 43.

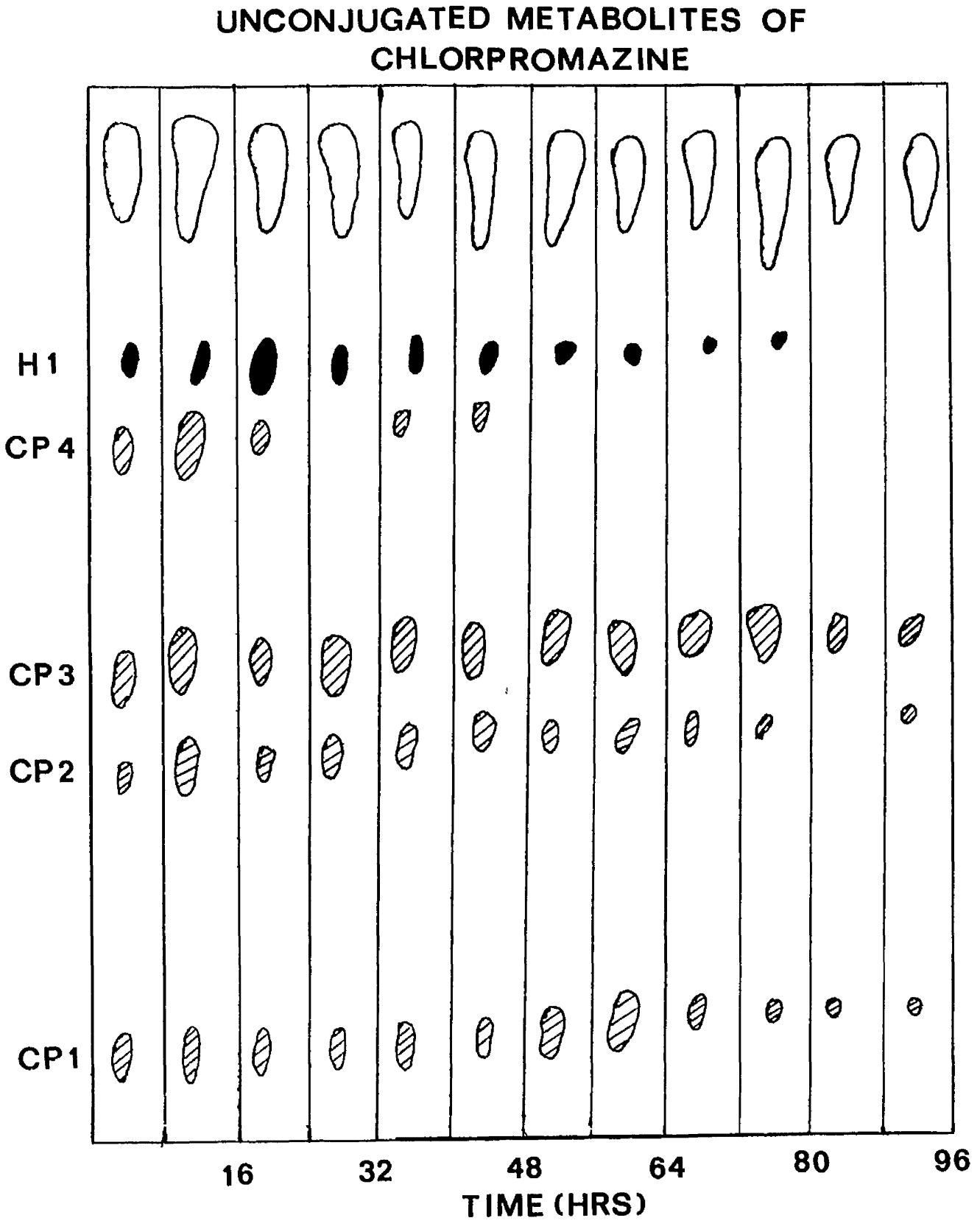

UNCONJUGATED METABOLITES OF
CHLORPROMAZINE
H1
CP4
CP3
CP2
CP1
16
32
48
64
80
96
TIME (HRS)

Table 13 Chromatographic, chemical and spectral properties of the Unconjugated Metabolites of Chlorpromazine.

Metabolite	Rf values			Colour Reactions					U.V. Max.
	System 1	System 2	System 3	H_2SO_4*	Ferric Chloride	Periodate	Ninhydrin	Nitroprusside	
CP1	0.09	0.05	0.68	A	-	-	-	-	270
CP2	0.37	0.12	0.74	A	-	-	-	-	270
CP3	0.46	0.14	0.80	A	-	-	-	-	270
Chlorpromazine Sulphoxide	0.47	0.16	0.80	A	-	-	-	-	270
CP4	0.67	0.24	0.85	A	+	-	-	-	250
Chlorpromazine	0.69	0.24	0.86	A	+	-	-	-	250
H1	0.73	0.29	0.87	B	+	+	-	-	250
7-hydroxy chlorpromazine	0.73	0.30	0.87	B	+	+	-	-	250

* A denotes pink colour reaction.

* B denotes purple colour reaction.

System 1 = Methanol/Acetic Acid/Water (5:3:2)
System 2 = Ethanol/Acetic Acid (1:1)
System 3 = Chloroform/Acetone/Diethylamine (2:7:1)

TABLE 13.

The ultra violet spectrum and Rf values of CP4 in each solvent system were identical to those of chlorpromazine. A positive reaction with the ferric chloride reagent confirmed that the ring sulphur atom was unoxidised,and there was no evidence of demethylation or hydroxylation. It was thus concluded that CP4 represented the parent compound. The other two compounds, (CP1, CP2,) were not fully identified. Both had ultra violet spectra corresponding to sulphoxide derivatives, and no reaction was observed with ferric chloride, periodate, ninhydrin or nitroprusside.

The remaining unconjugated metabolite, (H1), had a purple colour reaction with the sulphuric acid and periodate sprays, indicating hydroxylation had taken place. Its Rf values in the systems used, (0.73, 0.29, 0.87), corresponded to those of 7-hydroxy chlorpromazine, (0.73, 0.30, 0.87) as did its ultra violet spectrum. No reaction was noted with the ninhydrin or nitroprusside sprays. H1 was therefore identified as 7-hydroxy chlorpromazine.

Thus the major unconjugated metabolites of chlorpromazine in the horse are chlorpromazine sulphoxide, (CP3), 7-hydroxy chlorpromazine, (H1), the parent compound, (CP4), and two sulphoxide derivatives, (CP1, CP2), in which neither hydroxylation nor demethylation take place. Two other metabolites were detected in trace amounts, one of which was hydroxylated.

(b) Glucuronide Conjugated Metabolites.

Figure 44 shows a typical chromatogram following excretion of glucuronide conjugated metabolites after intramuscular administration

FIGURE 44.

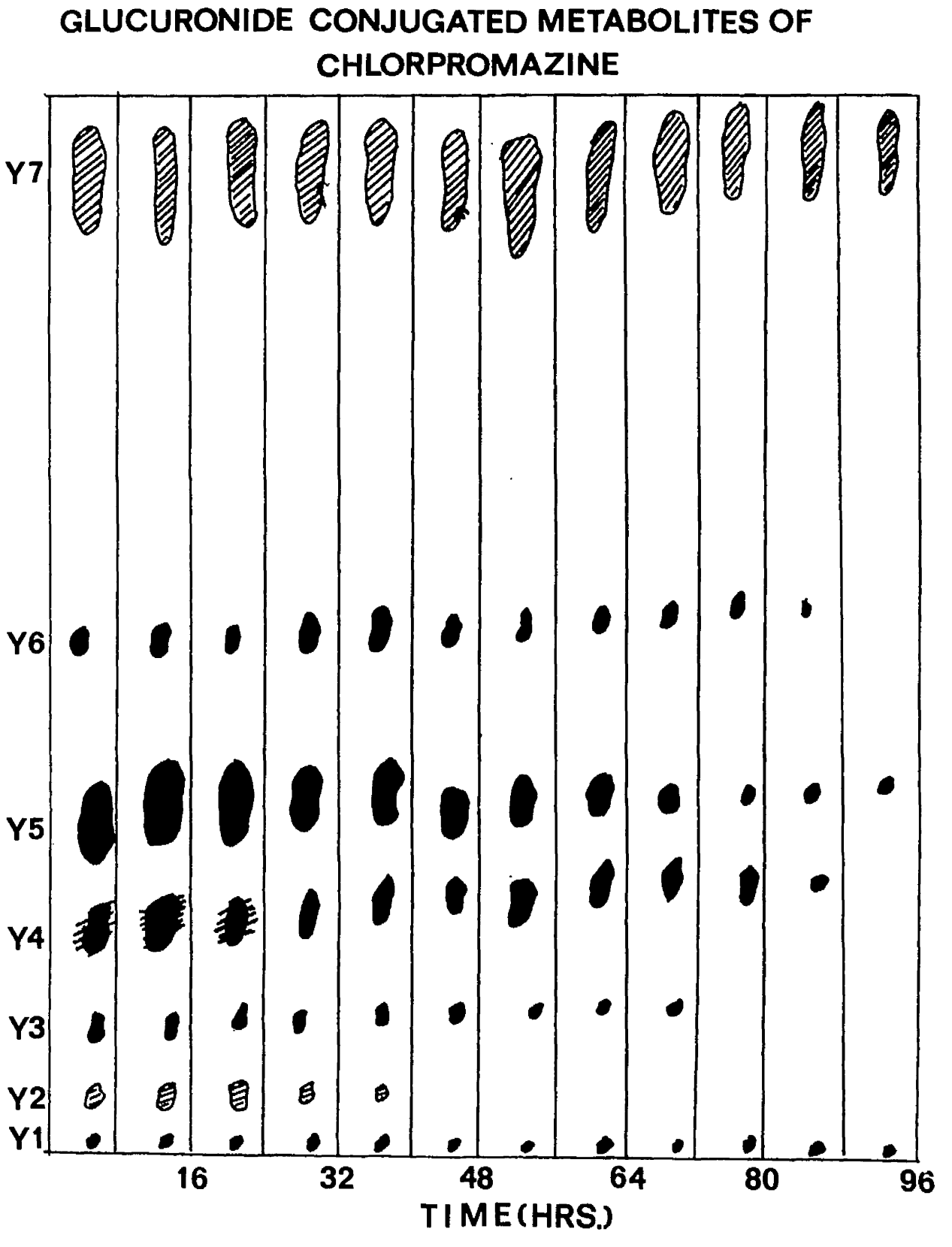

GLUCURONIDE CONJUGATED METABOLITES OF CHLORPROMAZINE
Y7
Y6
Y5
Y4
Y3
Y2
Y1
16
32
48
64
80
96
TIME(HRS.)

Table 14 Chromatographic, chemical and spectral properties of the glucuronide conjugated Metabolites of Chlorpromazine

Metabolite	Rf values			Colour Reactions					
	System 1	System 2	System 3	H_2SO_4*	Ferric Chloride	Periodate	Ninhydrin	Nitro-prusside	U.V. Max.
Y1	0.01	0.15	0.23	B	+	+	-	-	250
Y2	0.06	0.19	0.31	A	-	-	-	-	270
Y3	0.13	0.38	0.50	B	-	+	-	-	270
Y4	0.24	0.45	0.59	B	+	+	-	-	250
Y5	0.34	0.57	0.72	B	+	+	-	-	250
7-hydroxychlorpromazine	0.36	0.57	0.73	B	+	+	-	-	250
Y6	0.50	0.71	0.89	B	+	+	-	-	250
Y7	0.93	0.96	0.96	A	-	-	-	-	270

*A denotes pink colouration
*B denotes purple colouration

System 1 = Acetone/isopropanol/1% ammonia (9:7:4)
System 2 = Ethyl Acetate/Methanol/Diethylamine (7:2:1)
System 3 = Methanol/Acetic Acid/Water (5:3:2)

of chlorpromazine. As with promazine, the spots represent the genins obtained by enzymatic hydrolysis of the conjugated forms. Seven metabolites were found in this fraction. Spots Y1, Y3, Y4, Y5 and Y6 had a purple colour reaction with both sulphuric acid and periodate indicating that they were hydroxylated. Y2 and Y7, on the other hand, showed no evidance of hydroxylation.

Only one of these derivatives was fully identified. Spot Y3 ran to the same height in the solvent systems used, and had an identical ultra violet spectrum in 0.1N hydrochloric acid to 7-hydroxy chlorpromazine. There was no reaction with ninhydrin or or nitroprusside, but a reddish pink colouration was obtained with ferric chloride. Thus this metabolite was identified as 7-hydroxy chlorpromazine. Of the remaining hydroxylated compounds, Y1, Y4 and Y6 had ultra violet spectra corresponding to sulphide derivatives of chlorpromazine, and had a pink colour reaction with ferric chloride solution. However, they had no reaction with ninhydrin or nitroprusside. Using the same techniques it was concluded that spot Y3 corresponded to a sulphoxide derivative of chlorpromazine.

Compounds Y2 and Y7 had ultra violet spectra typical of sulphoxide derivatives of chlorpromazine but there was no evidence of hydroxylation or demethylation. As with promazine metabolite X5, conjugation in these compounds could take place through a side chain hydroxyl group which would have no reaction with the periodate spray. A summary of the results is given in Table 14.

(c) Sulphate Conjugated Metabolites.

Sulphate conjugated metabolites were found in only small

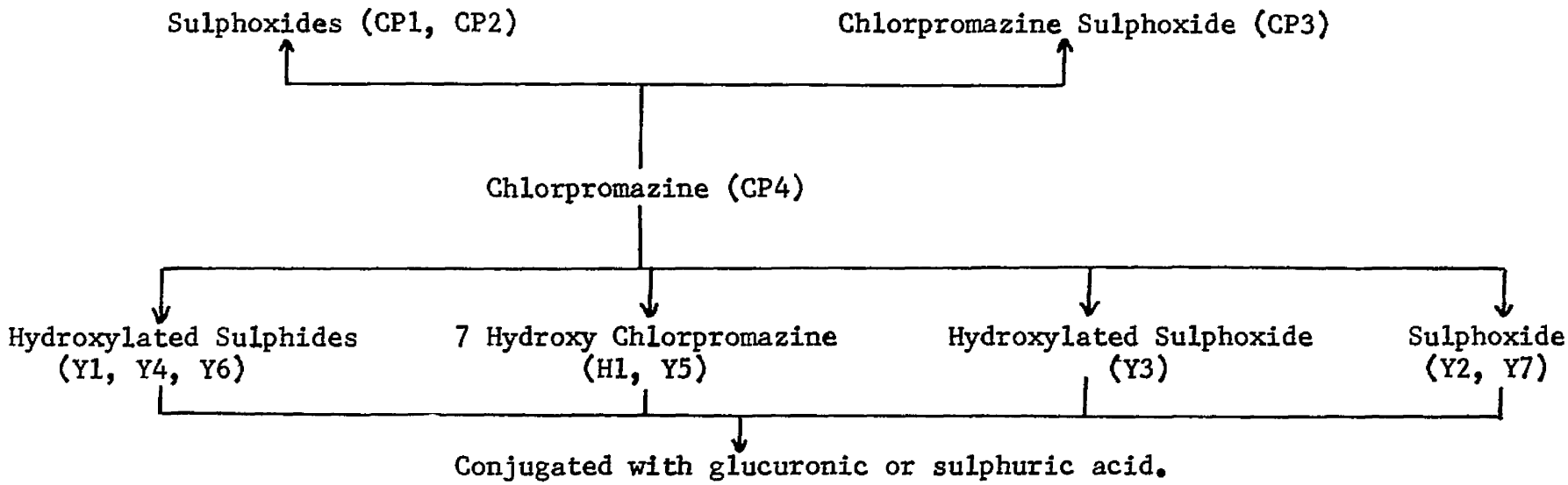


FIGURE 45.

Figure 45 Schematic diagram of metabolism of chlorpromazine by the horse.

quantities during the first 48 hours after intramuscular administration. Only four metabolites were detected in each sample which corresponded in chemical, spectral and chromatographic properties to compounds Y2, Y3, Y5 and Y7 found in the glucuronide fraction. Further analysis of sulphates using 2 litre volumes of pooled urine showed that compounds corresponding to Y4 and Y6 were also present.

(d) Oral Administration.

Oral administration gave similar metabolic patterns to those obtained after intramuscular injection, although a greater abundance of metabolites was noted in the sulphate fraction. Most samples contained five such metabolites corresponding in chemical, spectral and chromatographic properties to Y2, Y3, Y4, Y5 and Y7. Further analysis using larger samples of urine showed that all the genins obtained in the glucuronide fraction were also present as sulphate conjugates. A summary of metabolism of chlorpromazine by the horse is given in Figure 45.

(3) Metabolism of Acepromazine.

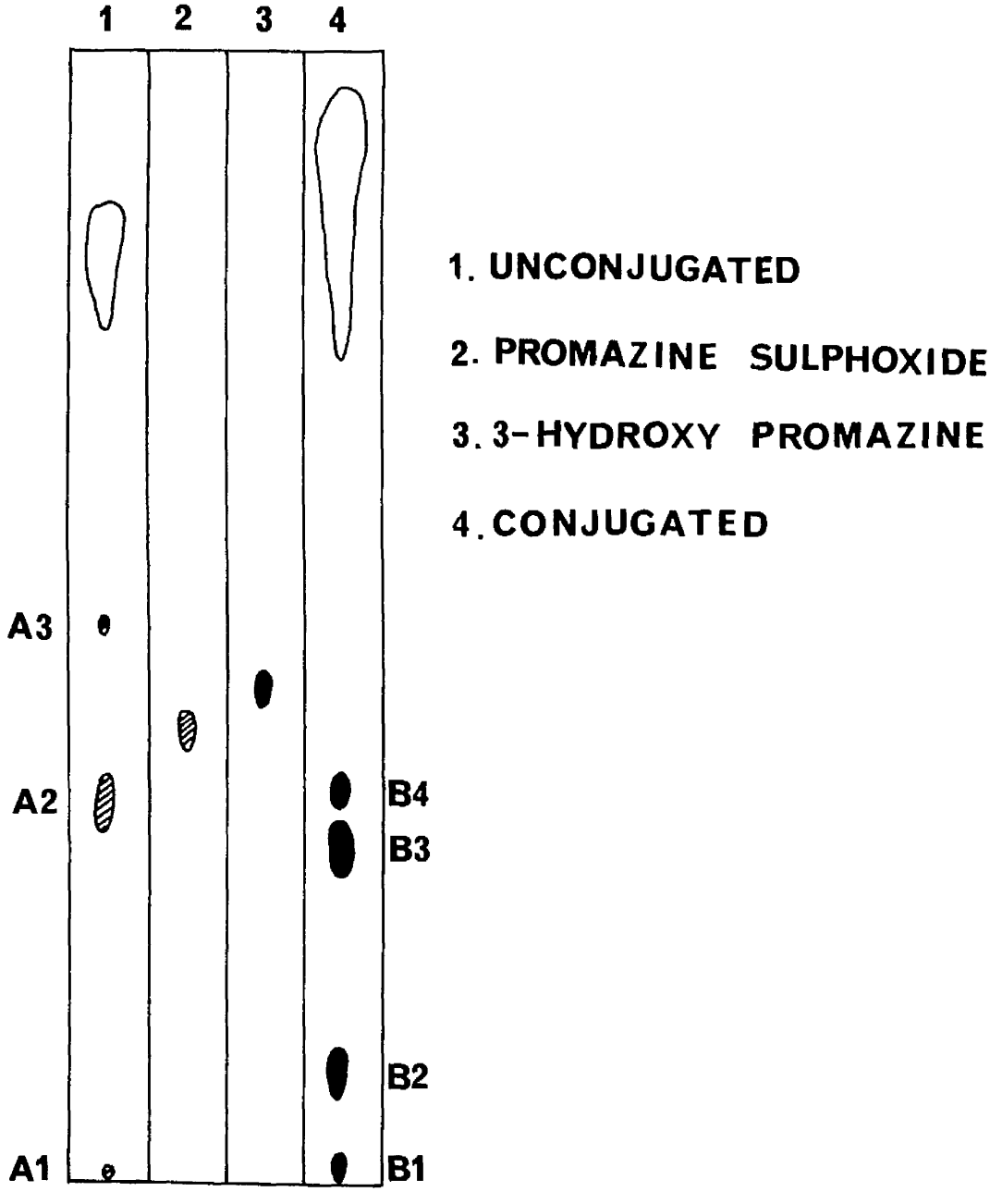
(a) Unconjugated metabolites.

Only one major metabolite, (A2), was found in the unconjugated fraction, present until 56 hours after intramuscular administration, and 72 hours after the oral route. However, two minor metabolites, (A1, A3), were occasionally found in small amounts. The chromatographic, chemical and spectral properties of the unconjugated fraction are recorded in Table 15.

Spot A2, on elution, produced an ultra violet spectrum

FIGURE 46.

METABOLITES OF ACEPROMAZINE.



1,2 = METHANOL – ACETIC ACID – WATER (5:3:2)

3,4 = ACETONE – ISOPROPANOL – 1% AMMONIA

Table 15 Chemical, Spectral and Chromatographic Properties of Metabolites of Acepromazine.

Metabolite	Rf values		Colour Reaction					
	System 1	System 2	H_2SO_4*	Ferric Chloride	Periodate	Ninhydrin	Nitroprusside	U.V. Max.
A1	0.01	-	A	-	-	-	-	270
A2	0.33	-	A	-	-	-	-	270
A3	0.49	-	A	-	-	-	-	270
B1	-	0.02	B	+	+	-	-	240,275
B2	-	0.10	B	-	+	-	-	250
B3	-	0.31	B	+	+	-	-	240,275
B4	-	0.35	B	+	+	-	-	240,275
Acepromazine Sulphoxide	0.50	-	A	-	-	-	-	270
Promazine Sulphoxide	0.40	-	A	-	-	-	-	270
3-Hydroxy Promazine	-	0.46	B	+	+	-	-	250

TABLE 15.

* A = Pink Colour Reaction
B = Purple Colour Reaction.

System 1 = Methanol/Acetic Acid/Water (5:3:2)
System 2 = Acetone/Isopropanol/1% Ammonia (8:7:5)

corresponding to a sulphoxide derivative of promazine. It had a pink colour reaction with sulphuric acid, but no reaction was observed with ninhydrin, nitroprusside, periodate or ferric chloride. It was thus identified as a sulphoxide derivative of promazine in which neither hydroxylation nor demethylation had taken place. Its Rf values in the solvent systems used were not comparable to those of promazine sulphoxide itself and due to the lack of standard reference compounds it was not further identified. Metabolites A1 and A3 had ultra violet spectra typical of sulphoxide derivatives of promazine.

They also produced a pink colour with 50% sulphuric acid, and no reaction with ferric chloride confirmed that they were in the sulphoxide form. Neither ninhydrin, nitroprusside nor periodate sprays produced any evidence of demethylation or hydroxylation.

Four metabolites, (B1 - B4), were detected in both the glucuronide and sulphate fractions up to 40 hours after administration Their chemical, spectral and chromatographic properties are summarised in Table 15. Each had a purple colour reaction with sulphuric acid and metaperiodate, indicating that hydroxylation had taken place. On elution, the ultra violet spectra of B1, B3 and B4 corresponded to that of acepromazine, showing that no oxidation of the nuclear sulphur atom had occurred, whereas that of B2 was typical of a sulphoxide derivative of acepromazine. This was confirmed using the ferric chloride spray, and the specific amine reagents showed no evidence of demethylation.

Thus acepromazine is metabolised by the horse to three

unconjugated (A1, A2, A3) and four conjugated derivatives (B1 - B4) occurring both as glucuronides and sulphates. A1, A2 and A3 are sulphoxide derivatives of promazine. B1, B3 and B4 are hydroxylated sulphide derivatives of acepromazine, whereas B2 is a hydroxylated sulphoxide. These routes of metabolism were also found in the dog. A chromatogram of the individual metabolites is shown in Figure 46.

(4) Metabolism of Propionylpromazine.

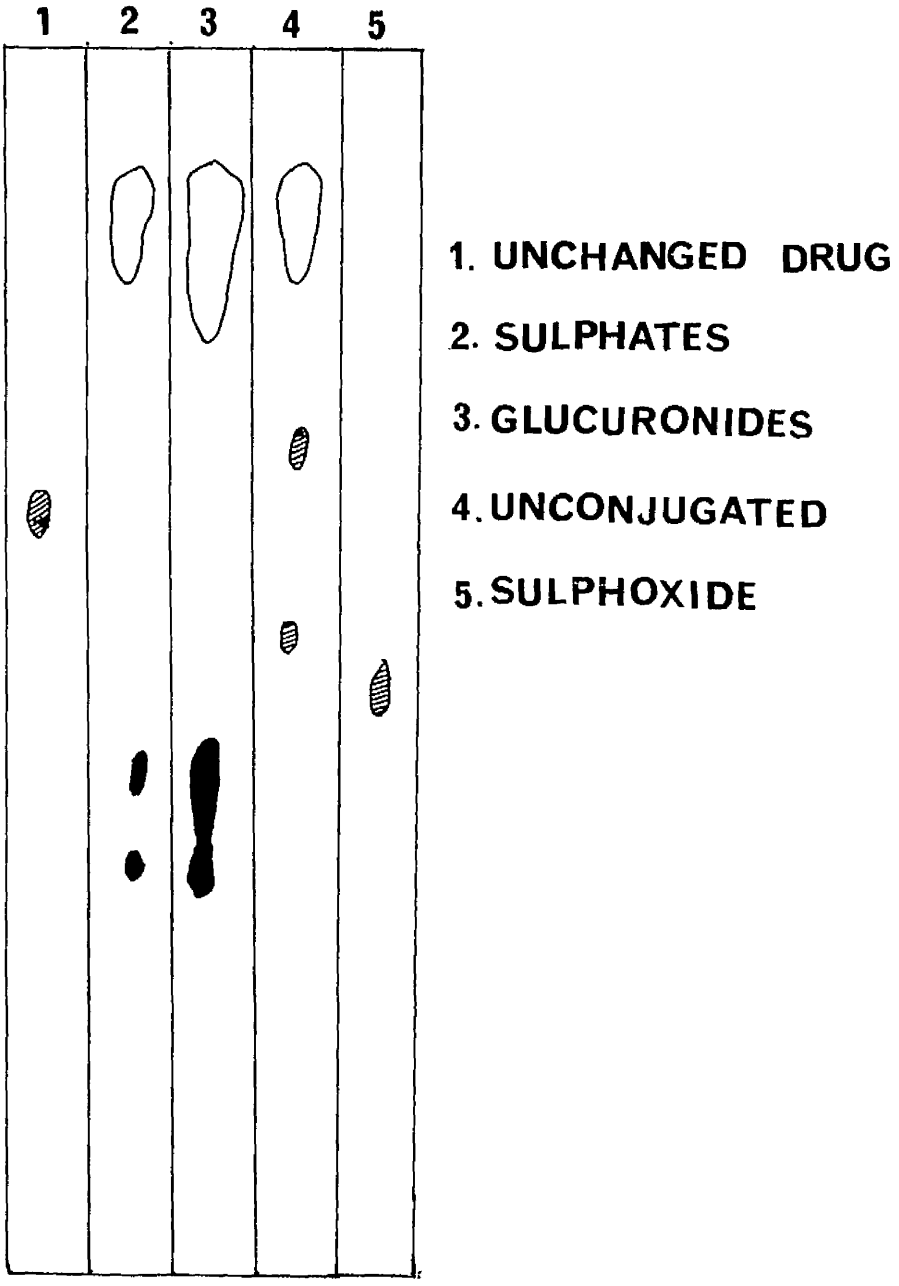
The major metabolites of propionylpromazine in the horse are shown in Figure 47. Two metabolites, (PR1, PR2), were found in the unconjugated fraction, which, on elution with 0.1N hydrochloric acid, produced ultra violet spectra corresponding to sulphoxi derivatives of promazine. They produced a reddish pink colouration with 50% sulphuric acid, but no reaction was observed with any of the other specific reagents.

Each conjugated fraction also contained two metabolites, (PH1, PH2), but neither could be eluted in sufficient amounts to produce an interpretable ultra violet spectrum. They had positive reactions with both sulphuric acid and periodate, and no evidence of demethylation was observed. Only PH2 had a positive reaction with the ferric chloride spray, showing that its nuclear sulphur atom was unoxidised.

The major metabolites of propionylpromazine in the horse are therefore two unconjugated sulphoxide derivatives of promazine which are neither hydroxylated nor demethylated, (PR1,PR2), and two hydroxylated metabolites, conjugated to either glucuronic

FIGURE 47.

I.M COMBELEN-QUALITATIVE EXCRETION PATTERNS



or sulphuric acid, one of which, (PH2), is a sulphide and the other, (PH1), a sulphoxide.

D. Limits of Detection of Urinary Metabolites.

Intramuscular doses of each drug, approximating to the minimum amount necessary to produce a noticeable effect, were administered over periods of 2 to 3 weeks to determine whether they were detectable at such levels. Details of the doses administered, volumes analysed, periods of collection, and metabolites detected are given for each drug.

(1) Promazine.

Promazine hydrochloride, (0.3, 0.5 or 0.7 mg/Kg as a 5% solution), was administered intramuscularly on nine days over a two week period. On the remaining days the horses received a control injection of saline. Urine was collected and pooled for approximately 6 hours after injection and separate aliquots of 300 ml were used for qualitative and quantitative analysis. From initial studies this was found to be the smallest volume from which metabolites could be detected using the spectroscopic technique. Details of the doses administered and results obtained using ultra violet and visible spectroscopy are shown in Table 16.

After injection of 0.5 or 0.7 mg/Kg. the unconjugated fraction was just detectable by ultra violet spectroscopy but they were not always detectable from the visible spectrum after reaction with sulphuric acid. No unconjugated metabolites were found using either form of spectroscopy after the 0.3 mg/Kg dose. Sulphate conjugates were found only occasionally in trace amounts.

Table 16 Results from ultra violet and visible spectra of extracts after intramuscular administration of small doses of promazine.

Day	Dose(mg/Kg)	Ultra Violet			Visible Spectrum		
		Unconjugated	Sulphates	Glucuronides	Unconjugated	Sulphates	Glucuronides
1	0.5	SO	-	-	-	-	-
2	0.7	SO	Trace	S	+	-	+
3	0.3	-	Trace	S	-	-	+
4	0	-	-	-	-	-	-
7	0.3	-	-	-	-	-	-
8	0.5	SO	-	-	-	-	-
9	0.7	SO	Trace	-	+	-	-
10	0.5	SO	Trace	-	+	+	+
11	0.7	-	-	S	-	+	+

SO = Sulphoxide spectrum S = sulphide spectrum

\+ = Visible spectrum obtained - = No spectrum obtained

TABLE 16.

Glucuronide conjugated metabolites were only detected occasionally in small concentration as sulphide derivatives. Howeve their appearance was not dependent on the dose administered. For example, after an initial dose of 0.5 mg/Kg. no glucuronides were detected, whereas doses of 0.7 mg/Kg and 0.3 mg/Kg on days 2 and 3 gave rise to weak ultra violet spectra typical of sulphide derivatives of promazine. This seemed to indicate a build up of metabolites,after successive daily dosage, but since their concentrations were so close to the limits of detection for the technique this could not be fully established.

Representations of thin layer chromatograms following excretion of individual metabolites after such doses are shown in Figures 48, 49 and 50. The greater sensitivity of this technique allowed detection of metabolites in most samples. The unconjugated fraction was found in every sample, but only three metabolites were detected corresponding in chemical, spectral, and chromatographic properties to P1, P2 (promazine sulphoxide) and P3 (Pg.$_{203}$). P1, and P2 occurred in most samples, but P3 was only detected on one occasion (Day 7).

Five glucuronide conjugated metabolites, corresponding to compounds X1 - X5 were detected in small concentration on days 10 and 11. However, over the rest of the experiment the number appearing was variable, ranging from 4 on the second day to none on day 8 (Figure 49).

Sulphates were detected in small concentration throughout the experiment. Six metabolites were detected on days 10 and 11,

FIGURE 48.

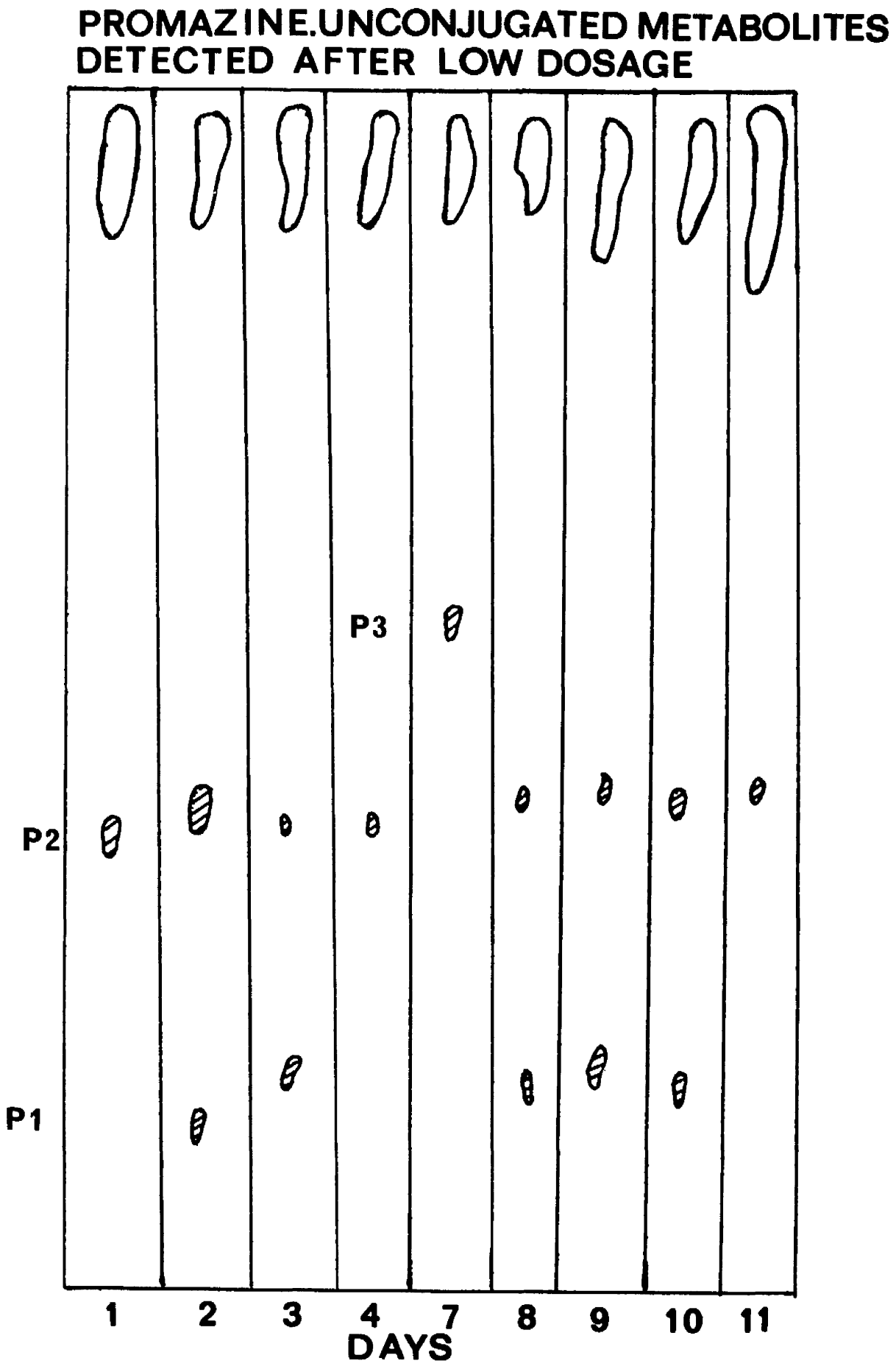

PROMAZINE.UNCONJUGATED METABOLITES
DETECTED AFTER LOW DOSAGE
P3
P2
P1
1
2
3
4
7
8
9
10
11
DAYS

FIGURE 49.

PROMAZINE. GLUCURONIDE CONJUGATED METABOLITES DETECTED AFTER LOW DOSAGE

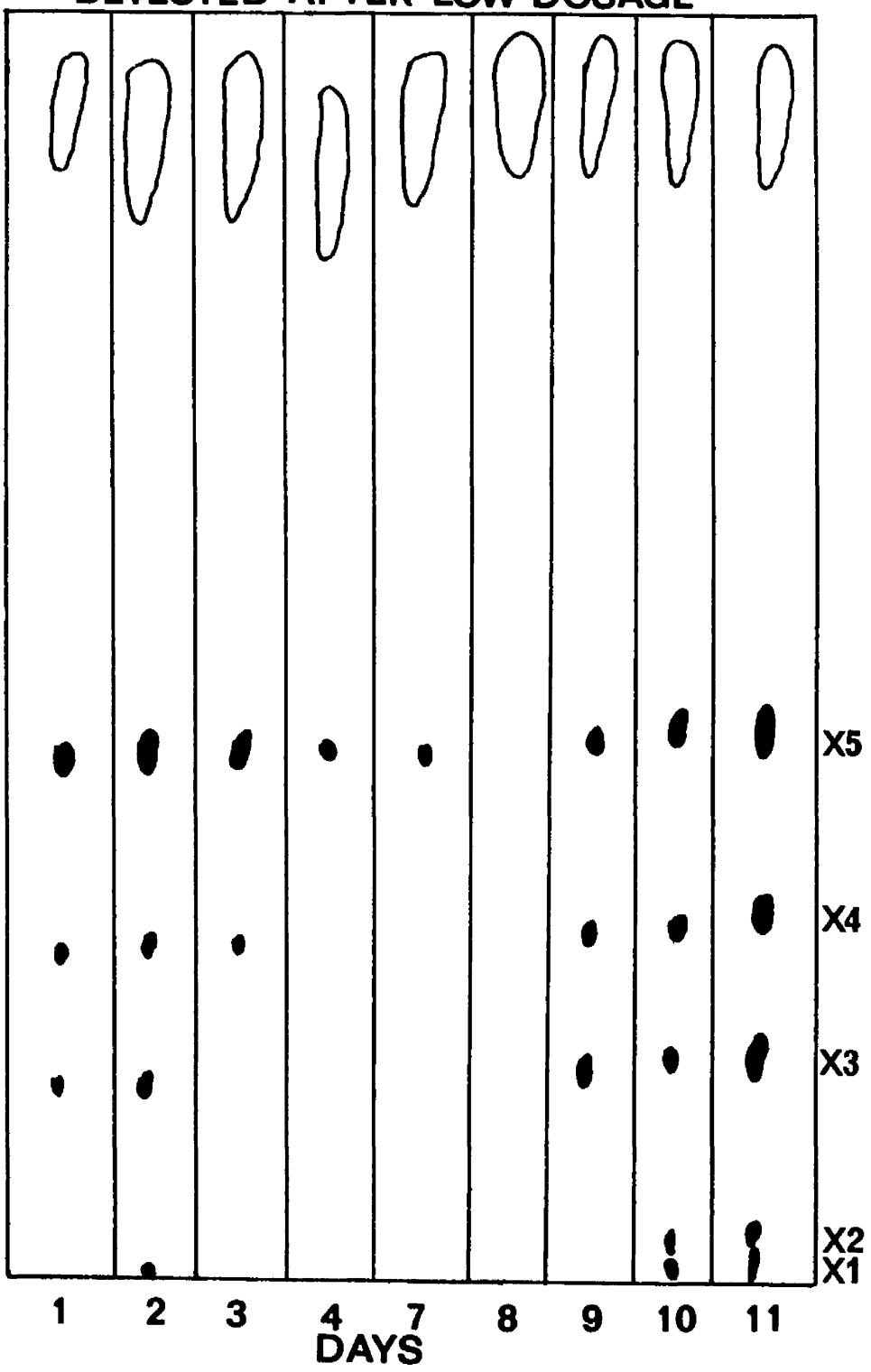
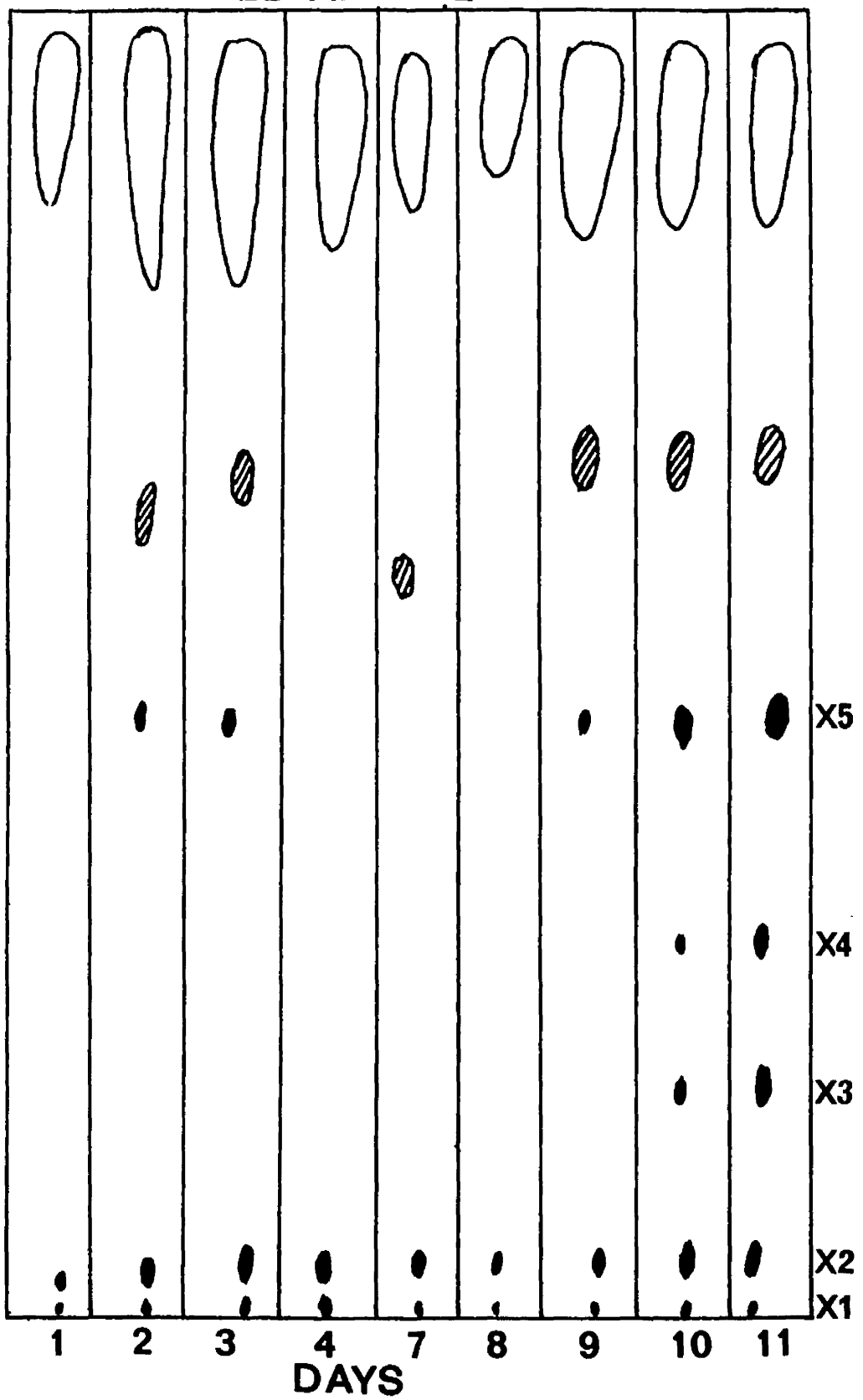


FIGURE 50.

PROMAZINE.SULPHATE CONJUGATED METABOLITES DETECTED AFTER LOW DOSAGE



five of which produced a purple colouration with sulphuric acid, and corresponded in physical and chemical properties to compounds X1 - X5. The sixth compound, (Rf = 0.65), had a reddish pink reaction with the sulphuric acid spray. Its ultra violet spectrum was typical of a sulphoxide derivative of promazine, and no colour was observed using periodate, ferric chloride, ninhydrin or nitroprusside solutions. It was thus concluded that this compound represented a sulphate derivative of promazine conjugated through nitrogen. It was detected on days 2, 3, 7, 9, 10 and 11. Apart from days 10 and 11, the number of sulphate conjugated metabolites found in any one sample varied between 2 and 4. Spots X1 and X2 were detected in every sample (Figure 50).

(2) Chlorpromazine.

A similar experiment was carried out after intramuscular administration of chlorpromazine hydrochloride, (0.1, 0.3 or 0.5 mg/Kg as a 5% solution). As for promazine 300 ml was found to be the smallest volume necessary for detection of metabolites by spectroscopy. The doses administered and results obtained by ultra violet and visible spectroscopy are shown in Table 17.

Concentrations of metabolites were again on the limits of detection. No unconjugated derivatives were found after doses of 0.1 mg/Kg and, with the exception of the initial dose (0.5 mg/Kg), where there was no evidence of metabolites, only small amounts were detected after the 0.3 or 0.5 mg/Kg doses. No sulphates were detected by this technique and glucuronide derivatives were only found in the first three samples, (0.5, 0.3, 0.1 mg/Kg respectively).

Table 17 Results from ultra violet and visible spectra of extract after intramuscular administration of small doses of chlorpromazine.

Day	Dose(mg/Kg)	Ultra Violet			Visible Spectrum		
		Unconjugated	Sulphates	Glucuronides	Unconjugated	Sulphates	Glucuronides
1	0.5	-	-	S	-	-	-
2	0.3	SO	-	S	+	-	+
3	0.1	-	-	S	-	-	+
4	0	-	-	-	-	-	-
7	0.1	-	-	-	-	-	-
8	0.3	-	-	-	-	-	-
9	0.5	SO	-	-	+	-	-
10	0.3	SO	-	-	+	-	-
11	0.3	SO	-	-	+	-	-

TABLE 17.

SO = Sulphoxide spectrum S = sulphide spectrum

\+ = Visible spectrum obtained - = No spectrum obtained

Chromatograms of individual metabolites are shown in figures 51, 52 and 53. Only two unconjugated metabolites were found corresponding in chemical, spectral and chromatographic properties to compounds CP3 (chlorpromazine sulphoxide) and CP4 (the parent drug). Either one or the other was present in most samples, but they were only found together on days 2, 10 and 11 (0.3 mg/Kg doses in each case).

Glucuronide conjugated metabolites were present in all samples. A maximum of four was detected on days 1, 2, 3, 10 and 11 corresponding in physical and chemical properties to compounds Y3, Y4, Y5 (7-hydroxychlorpromazine) and Y6. For the rest of the experiment the number of metabolites detected in each sample varied between 1 and 3. As in the unconjugated fraction the number of spots was not related to dose.

Six metabolites were found in the sulphate conjugated fraction. However, not more than four were detected in any one sample, and on three days (4, 7 and 11) no metabolites were present. Three spots had a purple colour reaction with sulphuric acid and corresponded chromatographically and chemically to compounds Y4, Y5 (7-hydroxy chlorpromazine), and Y6. The others, (Z1, Z2, Z3) produced a reddish pink colour with this reagent, having Rf values of 0.07, 0.17 and 0.33 respectively, and from their reaction with specific sprays appeared to be nonhydroxylated sulphoxide derivatives. Z1 was present in three samples (2, 3 and 9), whereas Z2 and Z3 were only found on days 10 and 1 respectively.

FIGURE 51.

CHLORPROMAZINE . UNCONJUGATED METABOLITES
DETECTED AFTER LOW DOSAGE

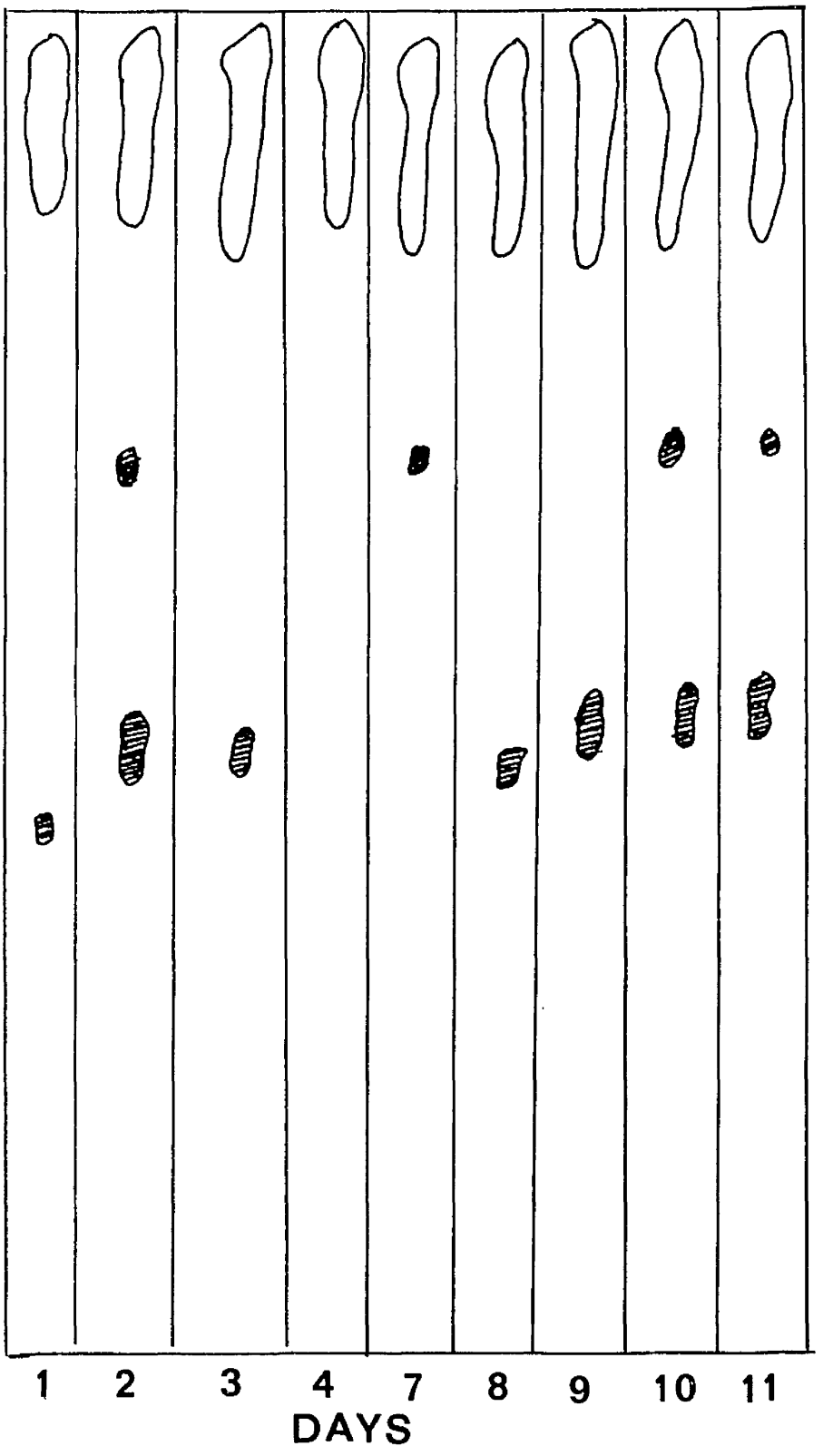


FIGURE 52.

CHLORPROMAZINE.GLUCURONIDE CONJUGATED METABOLITES EXCRETED AFTER LOW DOSAGE

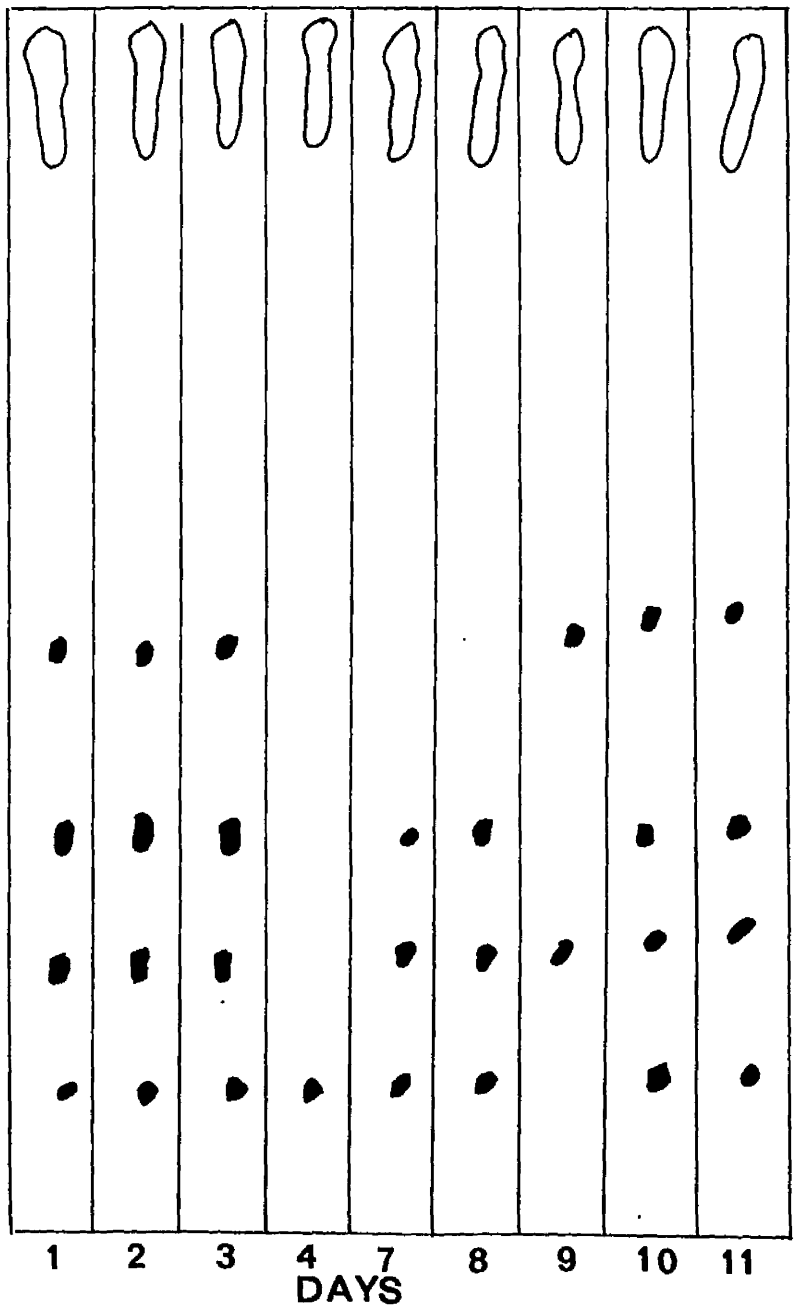
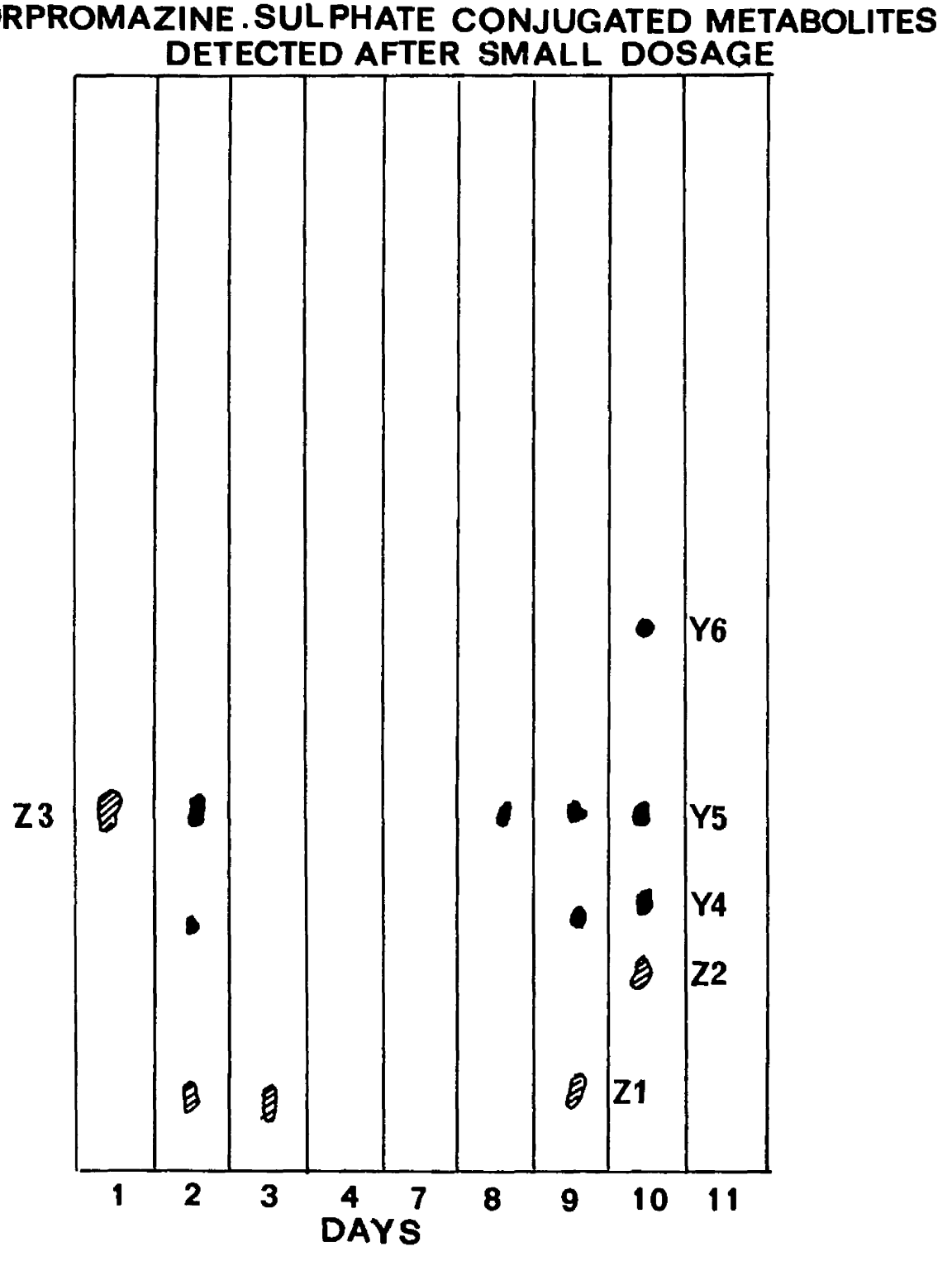


FIGURE 53.


CHLORPROMAZINE.SULPHATE CONJUGATED METABOLITES
DETECTED AFTER SMALL DOSAGE
Z3
Y6
Y5
Y4
Z2
Z1
1
2
3
4
7
8
9
10
11
DAYS

The chromatogram of sulphate conjugated metabolites is shown in Figure 53.

Thus for chlorpromazine, as with promazine, metabolites obtained from 300 ml of urine after such low doses were on the limits of detection of the spectroscopic and chromatographic procedures employed. The individual metabolites corresponded to those obtained after higher doses, although spots were much less intense. Small quantities of additional metabolites, which appeared to be nonhydroxylated sulphoxides, were occasionally found in the sulphate fraction.

(3) Acepromazine.

Intramuscular doses of acepromazine maleate, (0.02, 0.04 and 0.08 mg/Kg as a 1% solution), were administered to horse 6 on 7 days over a two week period. Saline was injected on control days Urine was collected and pooled over 6 hours after injection, and in this case 500 ml aliquots were used for qualitative and quantitative analysis. Ultra violet and visible spectra of extracts using this volume showed no evidence of phenothiazine derivatives, although spots were detected by thin layer chromatography However, using 1 litre of urine after doses of 0.08 mg/Kg, weak ultra violet spectra typical of sulphoxide derivatives of promazine were obtained from the unconjugated fraction.

Figures 54 and 55 show chromatograms following excretion of individual metabolites. Only one derivative was found in small amounts in the unconjugated fraction, which corresponded in

FIGURE 54.

ACEPROMAZINE. UNCONJUGATED FRACTION DETECTED AT LOW DOSE LEVELS

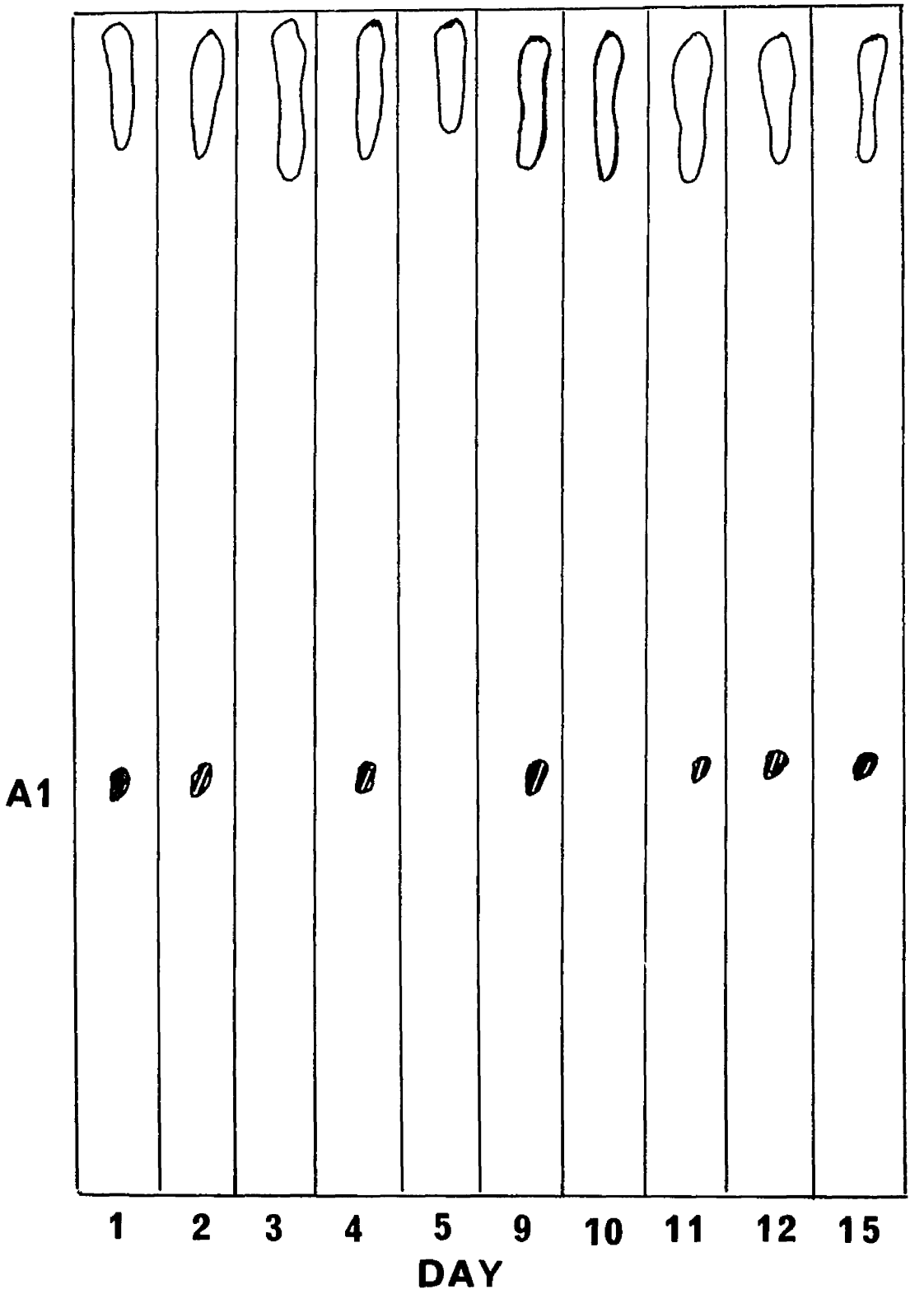
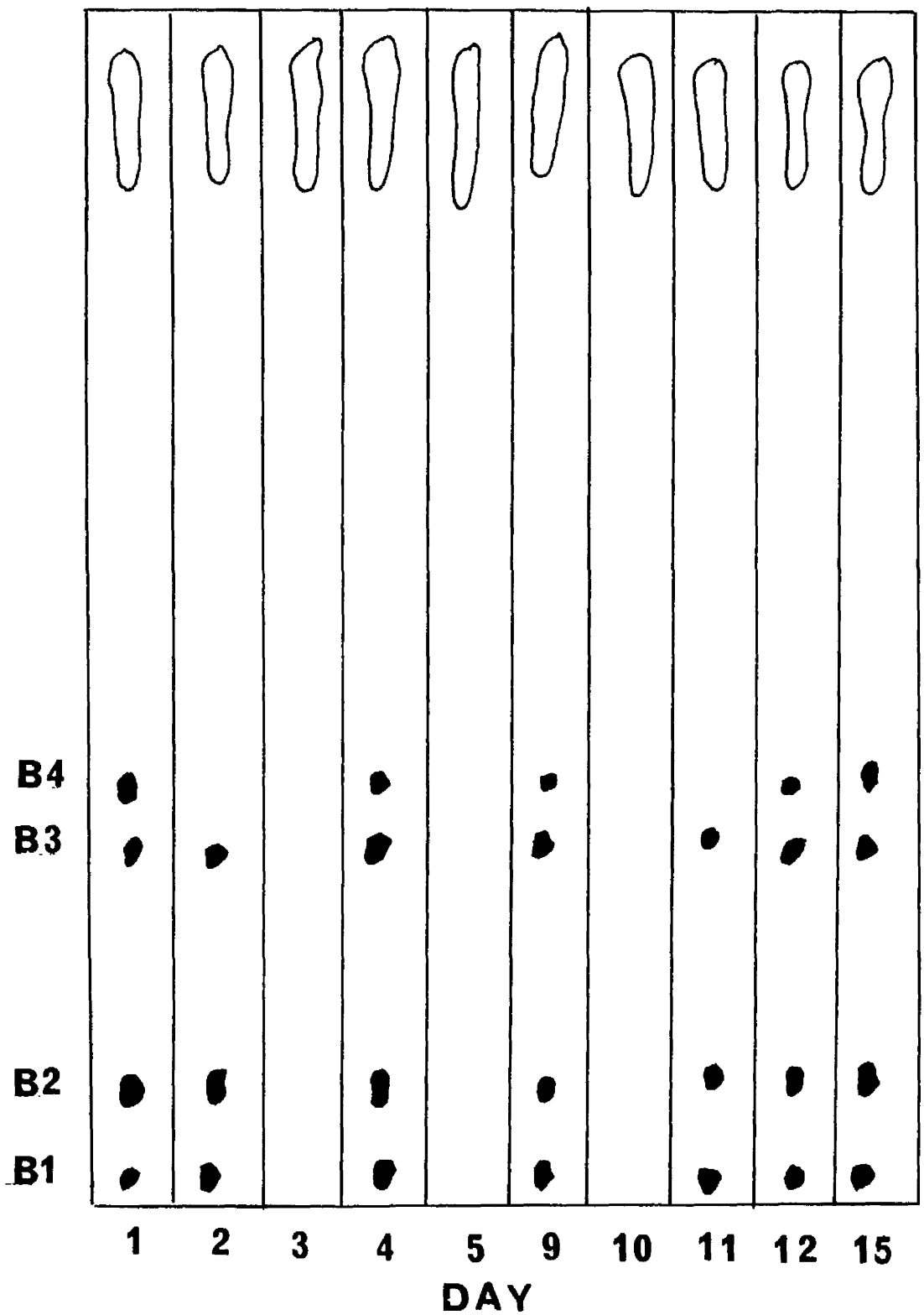


FIGURE 55.

ACEPROMAZINE.GLUCURONIDE FRACTION DETECTED AT LOW DOSE LEVELS



physical and chemical properties to metabolite A1. All four metabolites, found after larger doses (B1 - B4) were present in the glucuronide fraction. However, only B1 - B3 were detected after doses of 0.02 mg/Kg and no metabolites were detected after saline administration. B1 - B4 were also found in the sulphate fraction. Metabolites were detected in all three fractions on every day that the drug was administered.

E. Summary of Qualitative Results.

(1) Promazine.

In addition to the parent drug 30 metabolites were detected, nine of which were unconjugated derivatives. P2 was identified as promazine sulphoxide, P4 as the parent drug, and M1 as 3-hydroxypromazine. P1, P3 and P5 were sulphoxide derivatives showing no evidence of hydroxylation or demethylation and M1 and M2 were hydroxylated sulphide derivatives. Two other unconjugated metabolites, (Q1, Q2), appeared occasionally in small concentration and were not hydroxylated. 11 metabolites were detected in both glucuronide and sulphate fractions. X5 was identified as 3-hydroxy promazine, X1, X2 and X4 were hydroxylated sulphides, X3 was a hydroxylated sulphoxide, and X6 was a non-hydroxylated sulphoxide. No evidence of demethylation was found in any of the derivatives. The other five metabolites in each fraction (Pg.20§ appeared only occasionally in small concentration.

(11) Chlororomazine.

20 metabolites were detected in addition to the parent drug, each fraction accounting for 7 derivatives. In the unconjugated

fraction CP3 was identified as chlorpromazine sulphoxide, CP4 as the parent drug, H1 as 7-hydroxy chlorpromazine, and CP1 and CP2 as sulphoxide derivatives which were neither hydroxylated nor demethylated. The other two unconjugated metabolites, (CQ1, CQ2), appeared only occasionally in small concentration. CQ2 appeared to be hydroxylated. In the glucuronide and sulphate fractions Y5 was identified as 7-hydroxy chlorpromazine, Y1, Y4 and Y6 were hydroxylated sulphides, and Y3 a hydroxylated sulphoxide. On the other hand Y2 and Y7 showed no evidence of hydroxylation.

(111) Acepromazine and Propionylpromazine.

3 unconjugated metabolites (A1, A2, A3) were detected as sulphoxide derivatives of promazine in which neither hydroxylation nor demethylation had taken place. Four metabolites were found in both the glucuronide and sulphate fractions. B1, B3 and B4 were hydroxylated sulphides, and B2 a hydroxylated sulphoxide derivative of acepromazine.

Six metabolites of propionylpromazine were detected, two in each fraction. The unconjugated derivatives, (PR1, PR2), were sulphoxide derivatives of promazine, and compounds PH1 and PH2, obtained after hydrolysis of either conjugated fraction were both hydroxylated. PH1 was in the sulphoxide form whereas PH2 was unoxidised.

Using small doses sufficient to produce an effect, metabolites could just be detected from 300 ml of urine. In the case of acepromazine, 1 litre of urine was required before metabolites could be detected by spectroscopy.

F. Gas Chromatography and Mass Spectroscopy.

Since many metabolites were not fully identified using thin layer chromatography with specific spray reagents and ultra violet spectroscopy, the possibility of using the much more sensitive techniques of gas liquid chromatography and mass spectroscopy for further structural determination was investigated.

(1) Stainless Steel Columns.

Initial studies were carried out on the series of reference compounds available using stainless steel columns. Optimum working temperatures were decided upon from a series of preliminary experiments, (Pg. 120). However, each compound produced more than one peak which was thought to be due to thermal decomposition catalysed by the metal walls of the column (Pg. 83).

On injecting 0.25 µl of a solution of promazine hydrochloride in methanol (7.5 mg/ml) at a column temperature of 250°C, two peaks were obtained with retention times of 0.8 and 1.7 minutes. Since chlorpromazine had previously been reported to decompose to 2-chloro-phenothiazine during gas chromatography (Gudzinowicz, Martin and Driscoll, 1964), it was assumed that the peaks were due to partial thermal decomposition of promazine to phenothiazine, (i.e. loss of the nitrogen side chain). This was confirmed by chromatography of the solution under the same conditions after addition of a few milligrams of phenothiazine. Two peaks appeared corresponding to those obtained from the promazine hydrochloride solution alone, but the first (Rt = 0.8 minutes) was greatly enlarged. The identity of the decomposition product was later confirmed by mass spectrometry (Pg. 247). By comparison of

the area under each peak, the extent of decomposition was found to be approximately 20% over the range of operating temperatures studied (150 - 300°C).

Injection of 0.5 µl of promazine sulphoxide, (7 mg/ml in methanol) under the same conditions, gave rise to five chromatographic peaks with retention times of 0.8, 1.6, 1.7, 3.9 and 5.0 minutes. That at 0.8 minutes corresponded to the retention time of phenothiazine, and addition of a few milligrams of promazine hydrochloride to the sulphoxide solution caused an enlargement in the peaks at 0.8 and 1.7 minutes. Thus promazine sulphoxide was broken down thermally to 4 decomposition products on steel column, two of which were identified as promazine and phenothiazine. This was later confirmed by mass spectrometry. Decomposition was approximately 50% over the range of temperatures studied, (150-300°C).

Chlorpromazine and its sulphoxide were also thermally degraded on metal column. Using the same chromatographic conditions as for promazine the parent drug produced two peaks, (Rt = 1.2 and 2.4 minutes), which, as was later confirmed by mass spectrometry, corresponded to 2-chloro phenothiazine and chlorpromazine respectively. Decomposition was of the order of 20% over the temperature range 150 - 300°C. Three peaks (Rt = 1.2, 2.4 and 6.3 minutes) were obtained from the sulphoxide, the first two corresponding to 2-chloro phenothiazine and chlorpromazine respectively. This was also confirmed by mass spectroscopy. As with promazine sulphoxide the extent of decomposition was approximately 50% over the temperature range studied.

Other derivatives of promazine and chlorpromazine were also thermally degraded over this range. Chlorpromazine N-oxide produced two peaks (Rt = 1.2 and 2.6) at 250°C, the first corresponding in retention time to 2-chloro phenothiazine. Both monodemethylated promazine and chlorpromazine were also decomposed. Desmonomethyl promazine had peaks at 0.9 and 2.0 minutes, and the corresponding chlorpromazine derivative at 1.4 and 2.7 minutes. As with the parent drugs decomposition was of the order of 20%. However, in neither case did the peaks correspond to phenothiazine, or its 2-chloro derivative.

No satisfactory peaks were obtained from 3-hydroxy promazine or 7-hydroxy chlorpromazine on metal columns under such conditions. Instead tailing peaks were obtained at 0.8 and 1.2 minutes respectively, corresponding to phenothiazine and 2-chloro phenothiazine. Decomposition, in this case, appeared to be 100%.

Chromatography of acepromazine and its sulphoxide was also investigated on metal columns, and decomposition was again noted. 0.5 µl acepromazine (7 mg/ml in methanol) was injected at 250°C and two peaks (Rt = 2.9 and 5.0 minutes) were obtained. It was later shown using mass spectroscopy that the peak at 2.9 minutes corresponded to 2-acetyl phenothiazine. Chromatography of acepromazine sulphoxide under the same conditions gave rise to four peaks (Rt = 1.8, 2.9, 5.0 and 6.3 minutes), those at 2.9 minutes and 5.0 minutes corresponding to 2-acetyl phenothiazine and acepromazine respectively. For the parent drug the degree of decomposition was approximately 30% and, for the sulphoxide of the order of 50%.

By successive dilution of solutions of standard reference compounds, it was found that their limits of chromatographic detention for the conditions described were of the order of 1 μg.

(2) Epicote Treated Columns.

The degree of decomposition using metal columns would not allow a suitable separation, or identification, of individual metabolites extracted from biological fluids. It was thus attempted to prevent such reactions by coating the columns with a compound that would not catalyse decomposition. The compound employed was Epicote 1001 resin and the method of preparing the columns is described on Page 120.

However, on chromatographing the standard reference compounds, the resin was found to inhibit decomposition to only a small extent. The same number of peaks were found with approximately the same retention times for each compound as when using the untreated columns, but there was an overall decrease in the percentage decomposition. Approximately 12% of the parent drugs, chlorpromazine N-oxide, and the demethylated derivatives were decomposed. However, the extent of decomposition of the sulphoxides ($\sim$50%) and of the hydroxylated derivatives ($\sim$ 100%) remained of the same order.

(3) Glass Columns and Mass Spectroscopy.

Since it was not possible to obtain single chromatographic peaks using either metal columns or their pre-treated analogues, further experiments were initiated using an all glass system. Each standard reference compound was chromatographed using 5' x $\frac{1}{8}$"

glass columns packed with 1% SE-30 at a temperature of 200°C, which from preliminary experiments was found to be the optimum temperature for this system. Details of the conditions employed and preparation of the columns are described on Page 120. Using this system each compound produced only one peak.

Since only single peaks were obtained using glass columns in the Varian Aerograph instrument, the possibility of obtaining similar results using the glass columns of the LKB Gas Chromatograph-Mass Spectrometer was also investigated. This would allow determination of the molecular weight of individual metabolites and complement structural elucidation by ultra violet, thin layer chromatographic and chemical techniques. Details of the operating conditions of the instrument are described on Page 121.

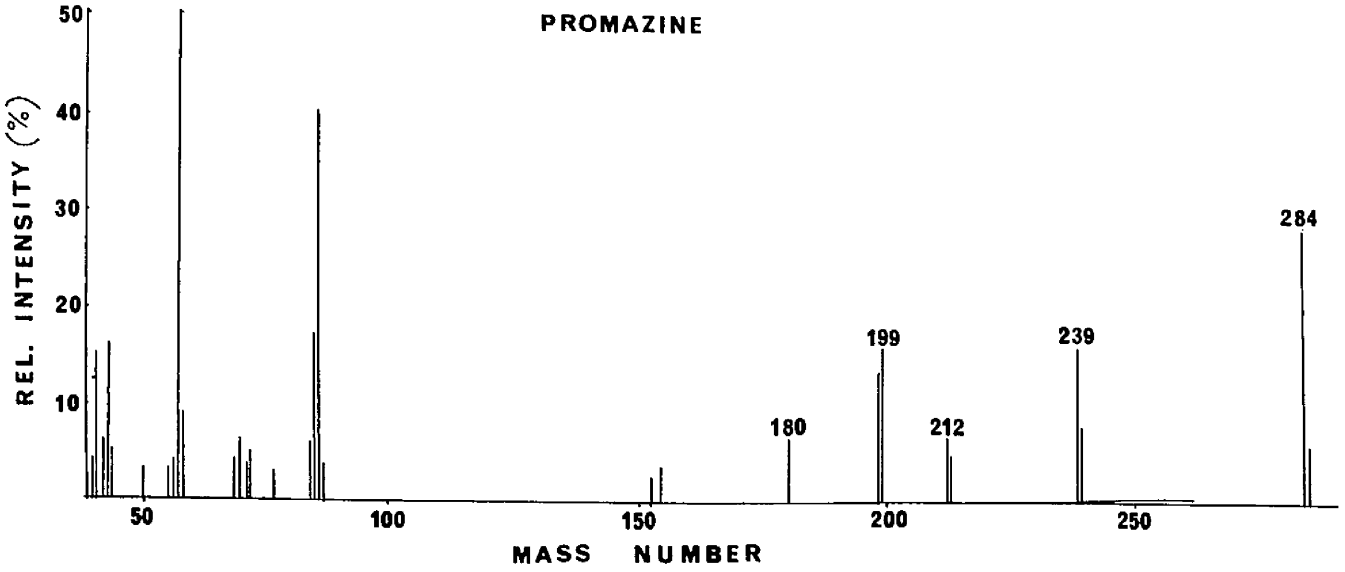
Each solution, having been run using glass columns on the gas chromatograph alone, was therefore subsequently run on the gas chromatograph-mass spectrometer, and, with the exception of sulphoxide derivatives, single peaks were obtained for each compound.

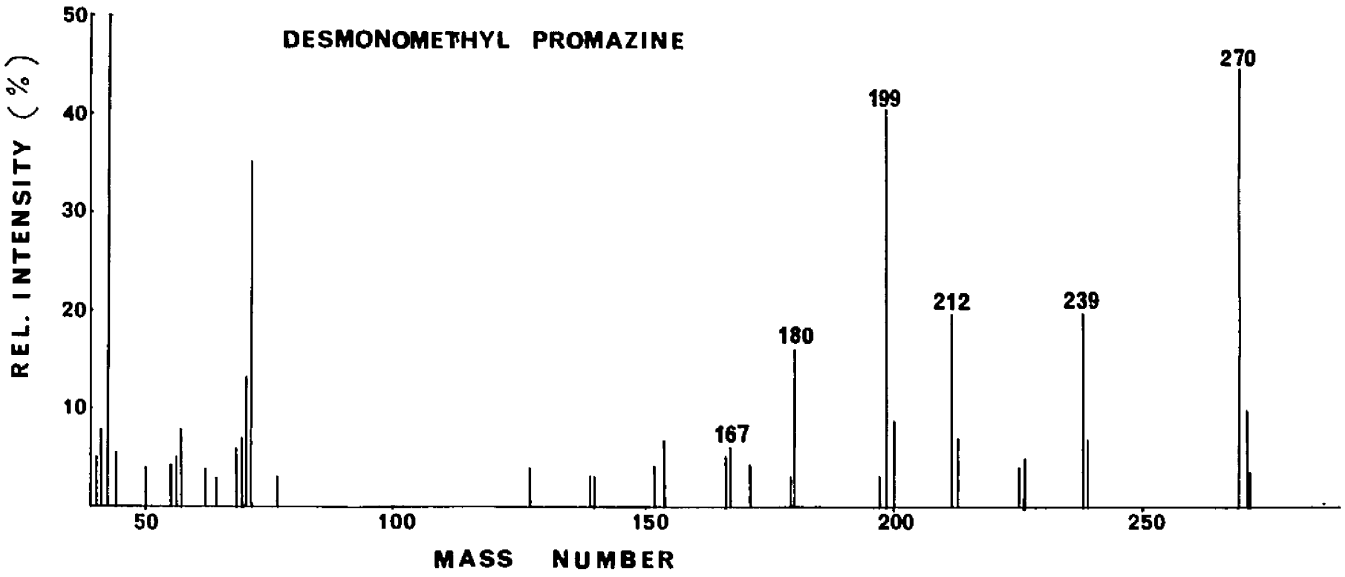
(a) Promazine.

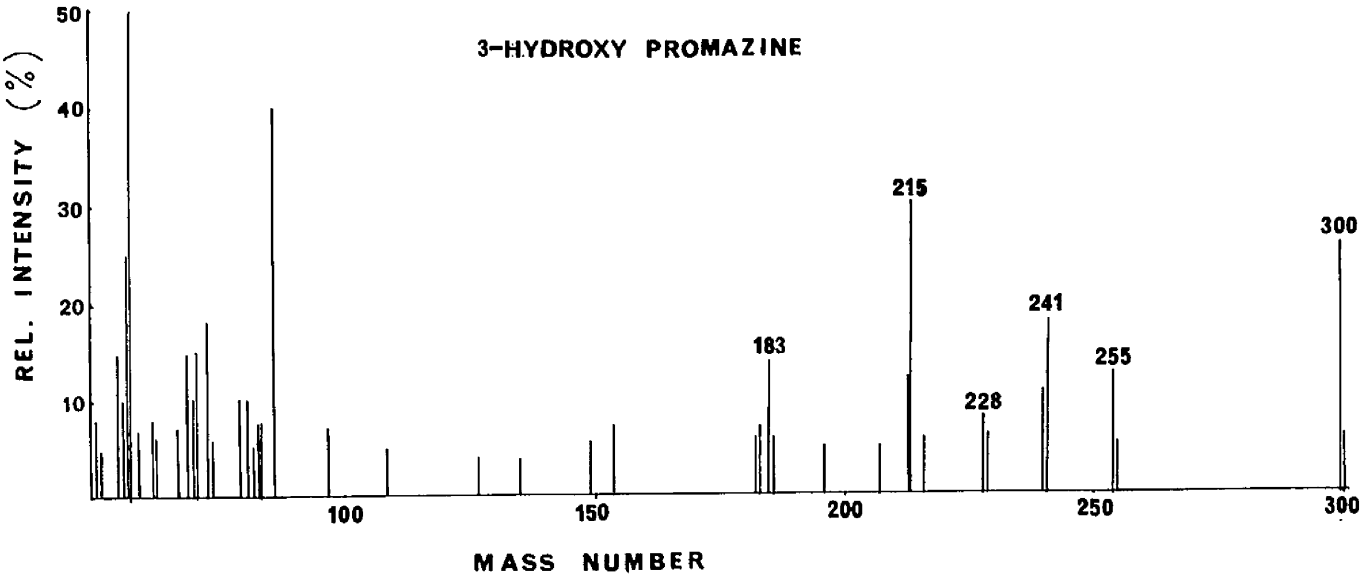
The mass spectrum of promazine obtained in this manner showed a molecular weight of 284. The mass numbers of the major breakdown fragments were 239, 225, 212, 199, 180, 167. A line diagram of the spectrum is shown in Figure 56.

Desmonomethyl-promazine also gave only one peak on the gas chromatograph-mass spectrometer, corresponding to a compound of molecular weight 270. The major breakdown fragments again appeared at 239, 225, 212, 199, 180, 167. The spectrum is

FIGURE 56


PROMAZINE
REL. INTENSITY (%)
50
40
30
20
10
284
199
239
180
212
50
100
150
200
250
MASS NUMBER


DESMONOMETHYL PROMAZINE
REL. INTENSITY (%)
50
40
30
20
10
270
199
212
239
180
167
50
100
150
200
250
MASS NUMBER


3-HYDROXY PROMAZINE
REL. INTENSITY (%)
50
40
30
20
10
215
300
241
183
255
228
100
150
200
250
300
MASS NUMBER

represented in Figure 56.

3-hydroxy promazine showed a molecular weight of 300. The mass numbers of the breakdown products in this case were exactly 16 mass units greater than the corresponding products from promazine being 255, 241, 228, 215, 196 and 163.

In the case of promazine sulphoxide three peaks were obtained and the instrument was temperature programmed from 150 to 200^{O}C at a rate of 5^{O}/minute to give adequate separation. The mass spectrum of the first peak, (Rt = 5.6 min), showed a molecular weight of 199 and a major breakdown product at 167, corresponding to the spectrum of phenothiazine (Figure 57). The second peak (Rt = 7.3 minutes), showed a molecular weight of 284 and major degradation products with mass numbers of 239, 225, 212, 199, 180 and 167 corresponding to the breakdown pattern of promazine (Figure 56a). The remaining peak (Rt = 8.7 minutes) had a molecular weight of 300 and the main products of degradation had mass numbers of 284, 255, 239, 225, 212, 199, 180, 167, which corresponded to the mass spectrum of promazine sulphoxide (Figure 58). Decomposition probably took place in the molecular separator between the two instruments.

Thus promazine and its derivatives have characteristic breakdown patterns in the mass spectrometer. Figure 59 shows a schematic representation of the compounds involved. It is postulated that promazine is broken down by progressive degradation of the side chain giving derivatives, F, I and C with mass numbers of 239, 225, and 212. Compound C then either loses the remainder of the side

FIGURE 56a

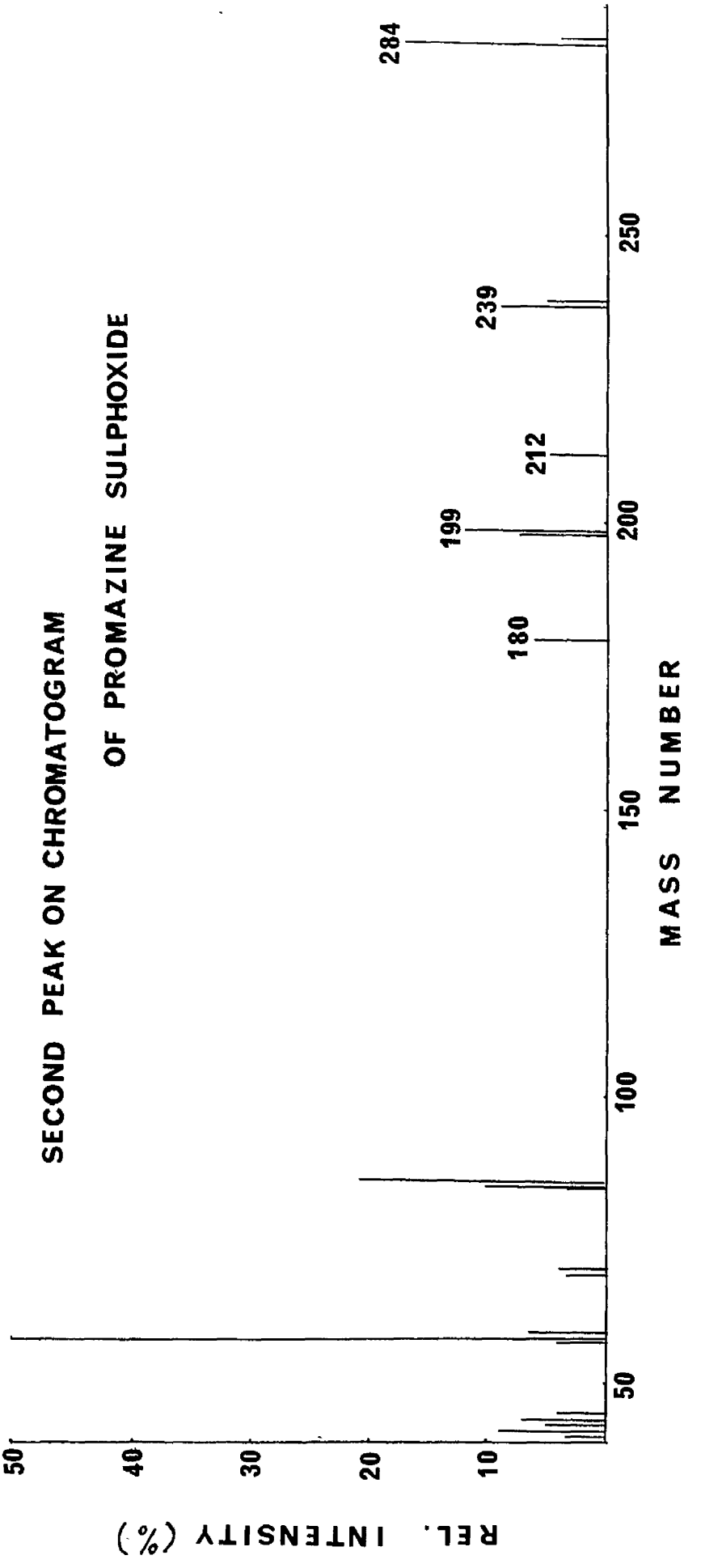
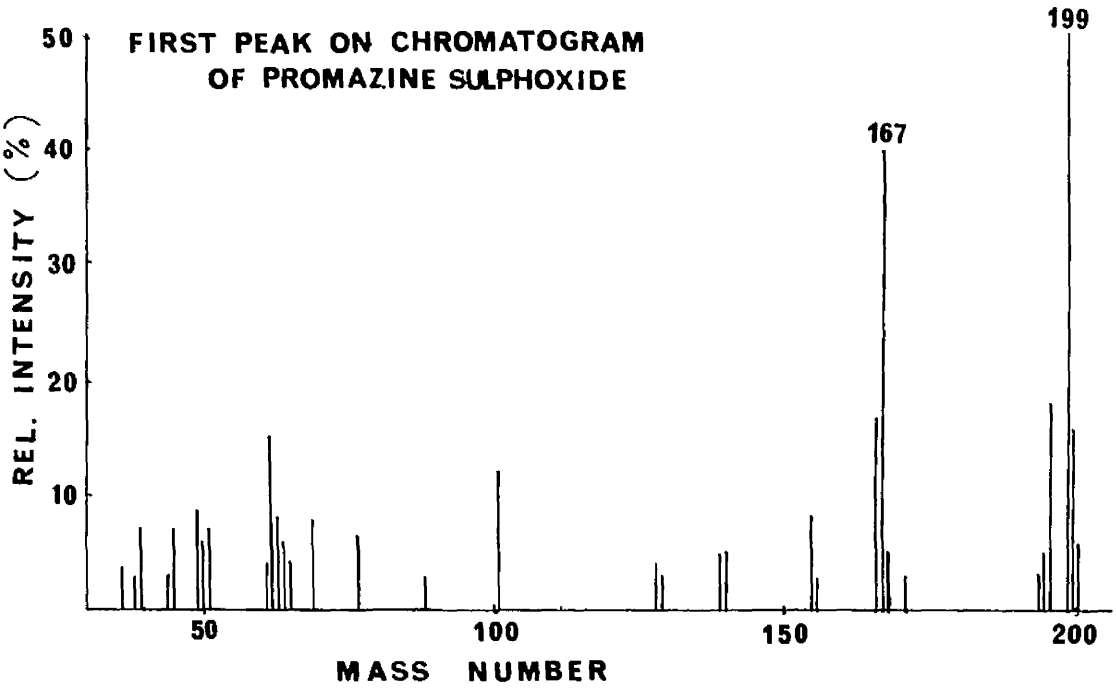

SECOND PEAK ON CHROMATOGRAM
OF PROMAZINE SULPHOXIDE
REL. INTENSITY (%)
50
40
30
20
10
MASS NUMBER
50
100
150
200
250
284
239
212
199
180

FIGURE 57.


FIRST PEAK ON CHROMATOGRAM
OF PROMAZINE SULPHOXIDE
199
167
50
40
30
20
10
REL. INTENSITY (%)
50
100
150
200
MASS NUMBER

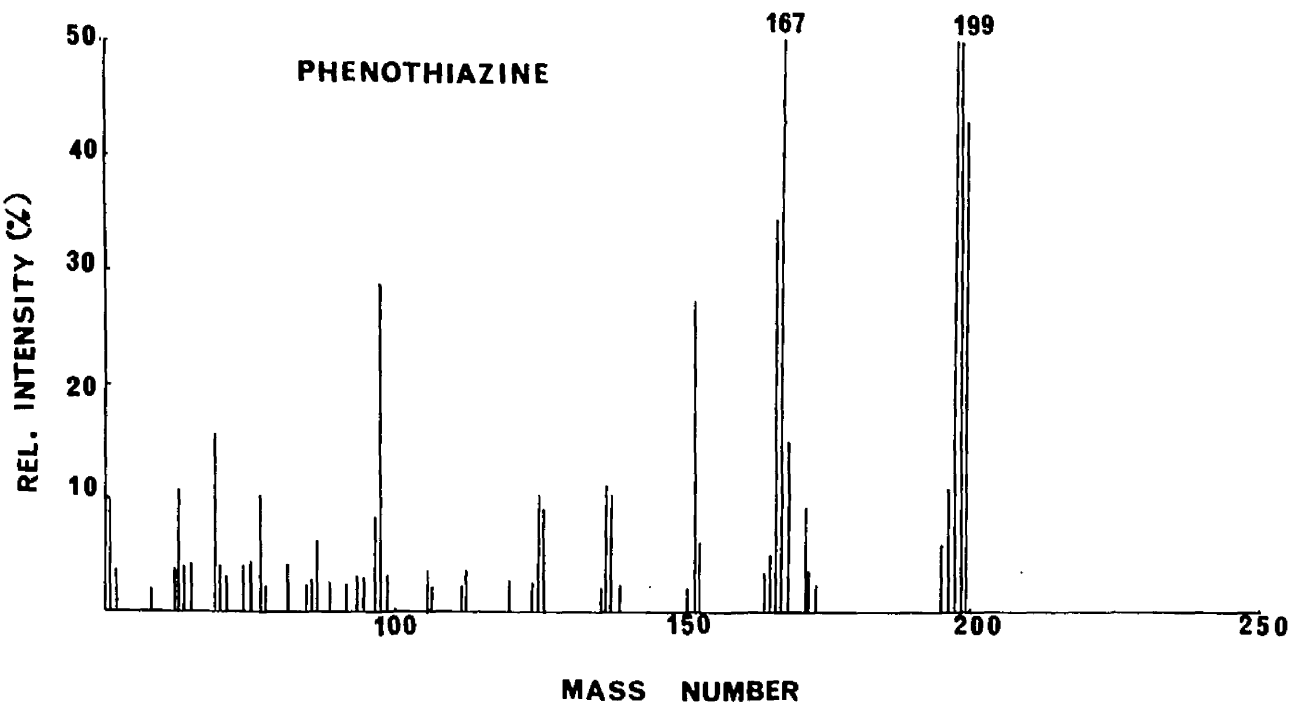
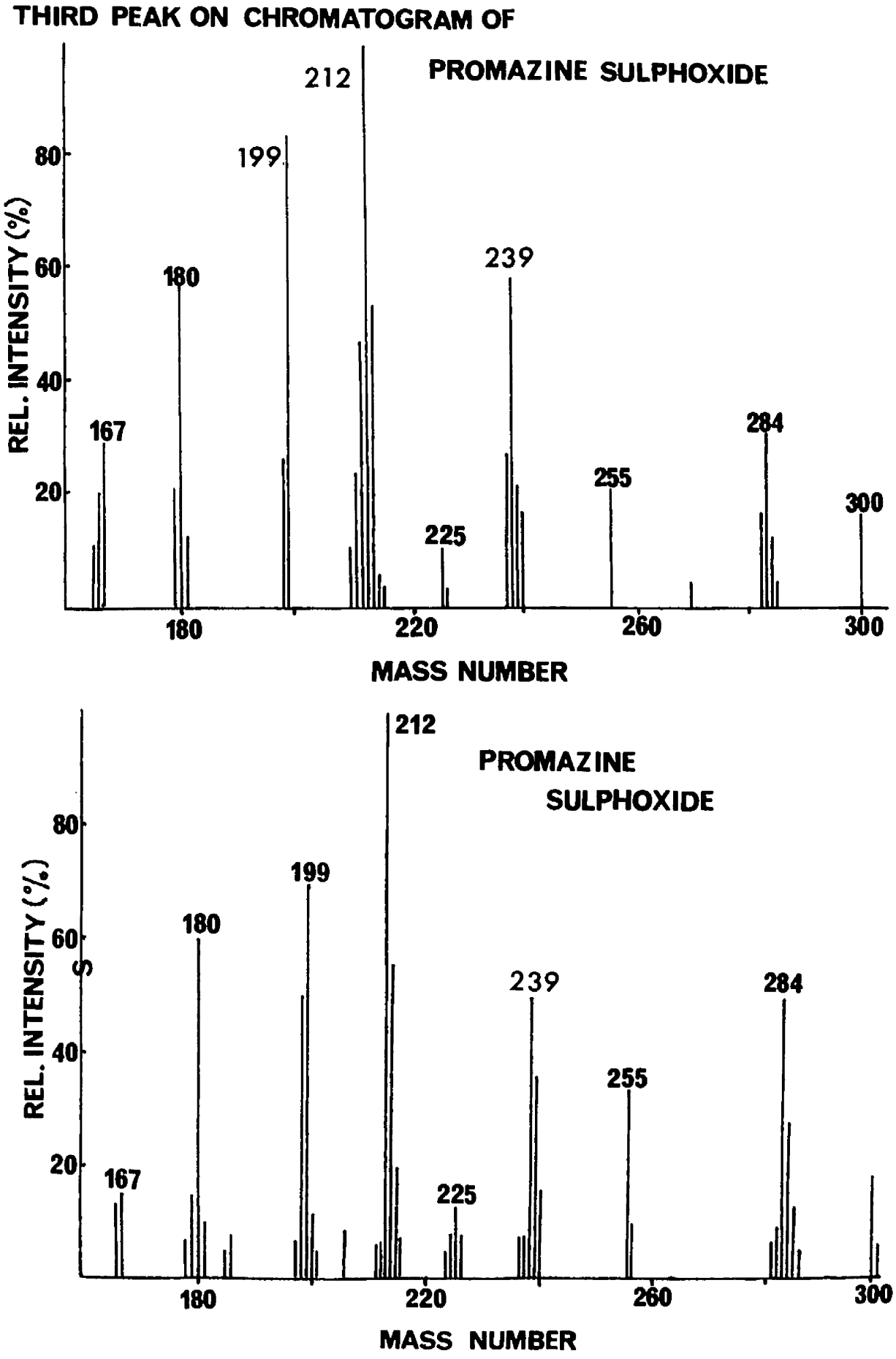

PHENOTHIAZINE
167
199
50
40
30
20
10
REL. INTENSITY (%)
100
150
200
250
MASS NUMBER

FIGURE 58


THIRD PEAK ON CHROMATOGRAM OF
PROMAZINE SULPHOXIDE
212
199
180
239
167
284
255
300
225
REL. INTENSITY (%)
80
60
40
20
180
220
260
300
MASS NUMBER
PROMAZINE
SULPHOXIDE
212
199
180
239
284
255
167
225
REL. INTENSITY (%)
80
60
40
20
180
220
260
300
MASS NUMBER

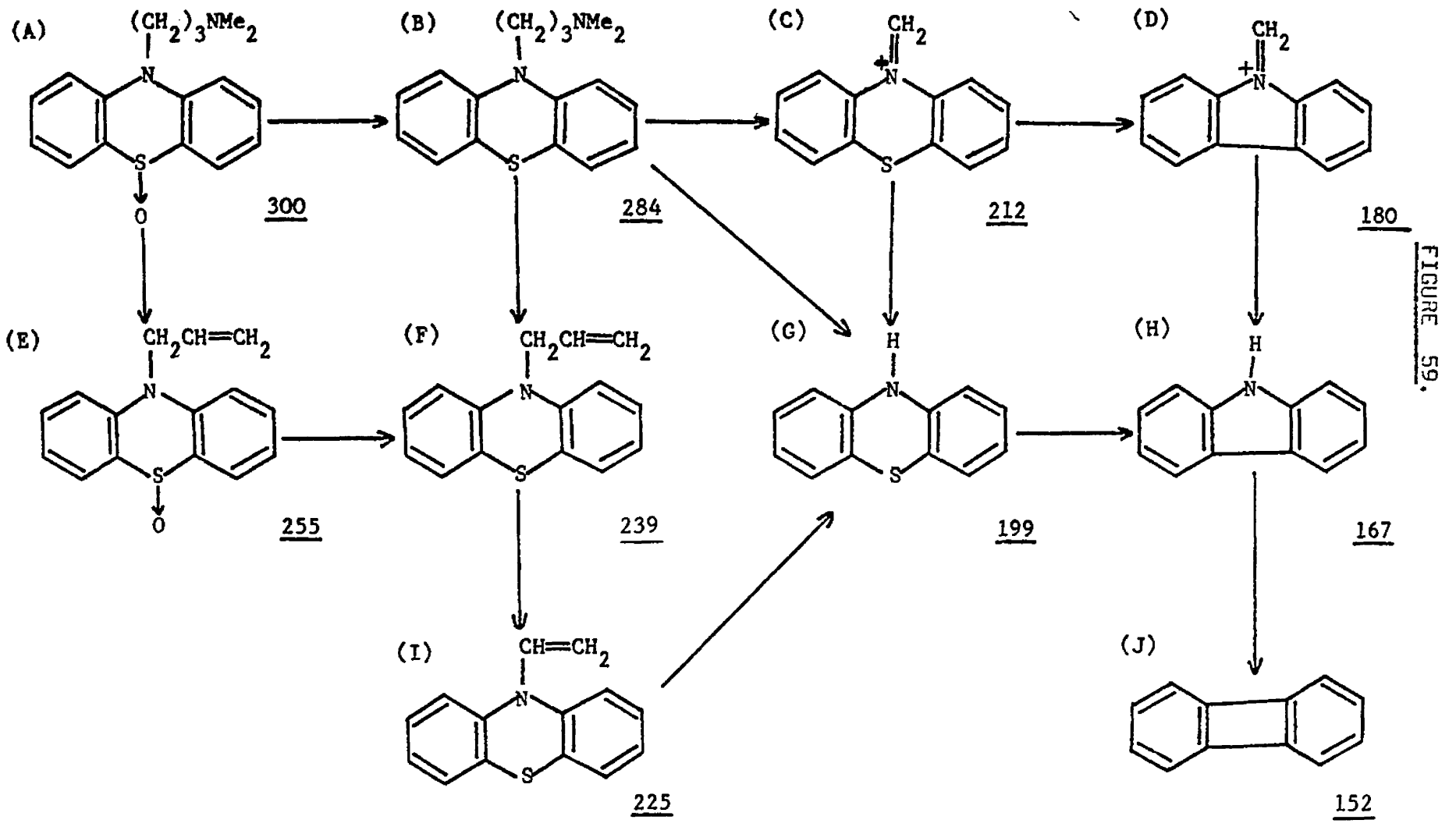


FIGURE 59.

Fig. 59. MASS SPECTRAL BREAKDOWN PATTERNS OF PROMAZINE AND PROMAZINE SULPHOXIDE.

chain to give phenothiazine (G), or loses the ring sulphur atom to give compound D, (180) G and D then lose the sulphur atom and the remainder of the side chain respectively, giving compound H (167), which further decomposes to compound J, (152).

Desmonomethylpromazine and, (by analogy with desdimethylchlorpromazine), desdimethylpromazine follow exactly the same breakdown pattern. Promazine sulphoxide is degraded to promazine and thereafter to the promazine derivatives C,D,F,G,H,I, and J. However, the oxygen atom can also remain during the first degradation of the side chain giving compound E (255), and is subsequently lost to give F (239). Thus the major derivatives found on the mass spectrum of promazine sulphoxide have mass numbers 300, 255, 239, 225, 212, 199, 180, 167. 3-hydroxy promazine, on the other hand, does not appear to lose the hydroxyl group until it is broken down to compound H. Due to the hydroxyl group, compounds analogous to C,D,F,G,H, and I are formed with a mass number 16 more than the corresponding promazine fragments. The mass numbers of the major breakdown products of the hydroxy derivative are 300, 255, 241, 228, 215, 196, 183.

Apart from molecular weight considerations, promazine and its derivatives are therefore also readily recognisable from the characteristic patterns of their mass spectra. As will be described (Pg. 253), the main hindrances to using the gas chromatograph-mass spectrometer for metabolic studies on such compounds are decomposition of sulphoxides, and difficulty in purification of the samples to a sufficient extent to give identifiable peaks.

(b) Chlorpromazine.

Chlorpromazine has a mass spectrum showing a molecular weight of 318, and the masses of the major breakdown products are 273, 259, 246, 233, 214 and 199. By subtracting 34 mass units from each of those numbers, (as would be the case if the chlorine was replaced by hydrogen), the pattern would be 284, 239, 225, 212, 199 and 180 which correspond to promazine derivatives A,F,I,C,G and D. Thus the breakdown of chlorpromazine in the mass spectrometer proceeds in a similar fashion to promazine, (Figure 60).

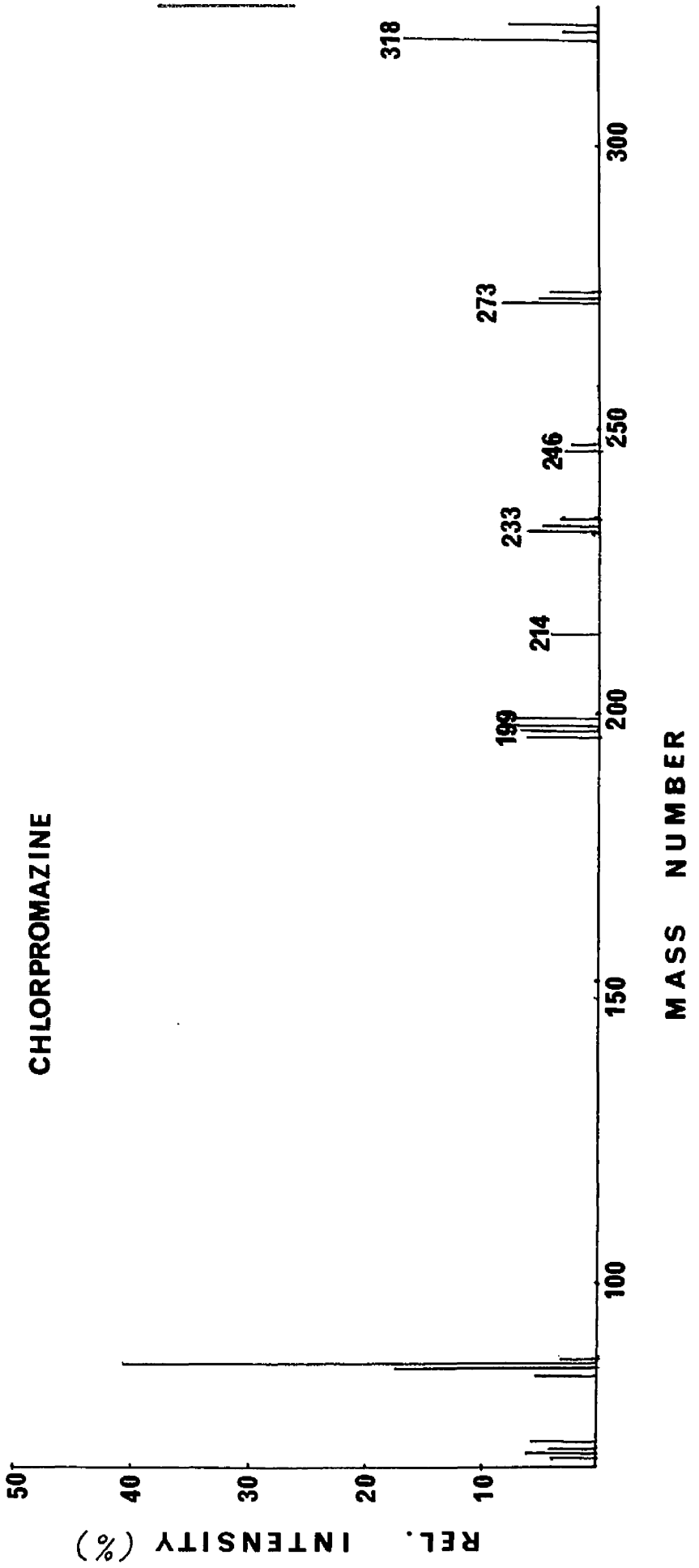
The respective molecular weights obtained for desmonomethyl- and desdimethylchlorpromazine were 304 and 290, and in both cases the mass numbers of the major degradation derivatives were 273, 259, 246, 233, 214 and 199.

Chlorpromazine N-oxide had a mass spectrum with breakdown fragments of mass numbers 334, 318, 273, 259, 233, 214 and 199.

As with 3-hydroxy promazine, 7-hydroxychlorpromazine also appeared to retain the hydroxyl function during most of the degradation process. Derivatives in the breakdown pattern had mass numbers of 334, 289, 275, 262, 249, 230 and 201.

Three peaks were obtained on the chromatogram of chlorpromazine sulphoxide. At 250°C their retention times were 0.8, 1.3 and 2.9 minutes. The first peak represented a compound of molecular weight 233, with a major degradation product of mass number 201. This

FIGURE 60


CHLORPROMAZINE
REL. INTENSITY (%)
50
40
30
20
10
MASS NUMBER
100
150
200
250
300
318
273
246
233
214
199

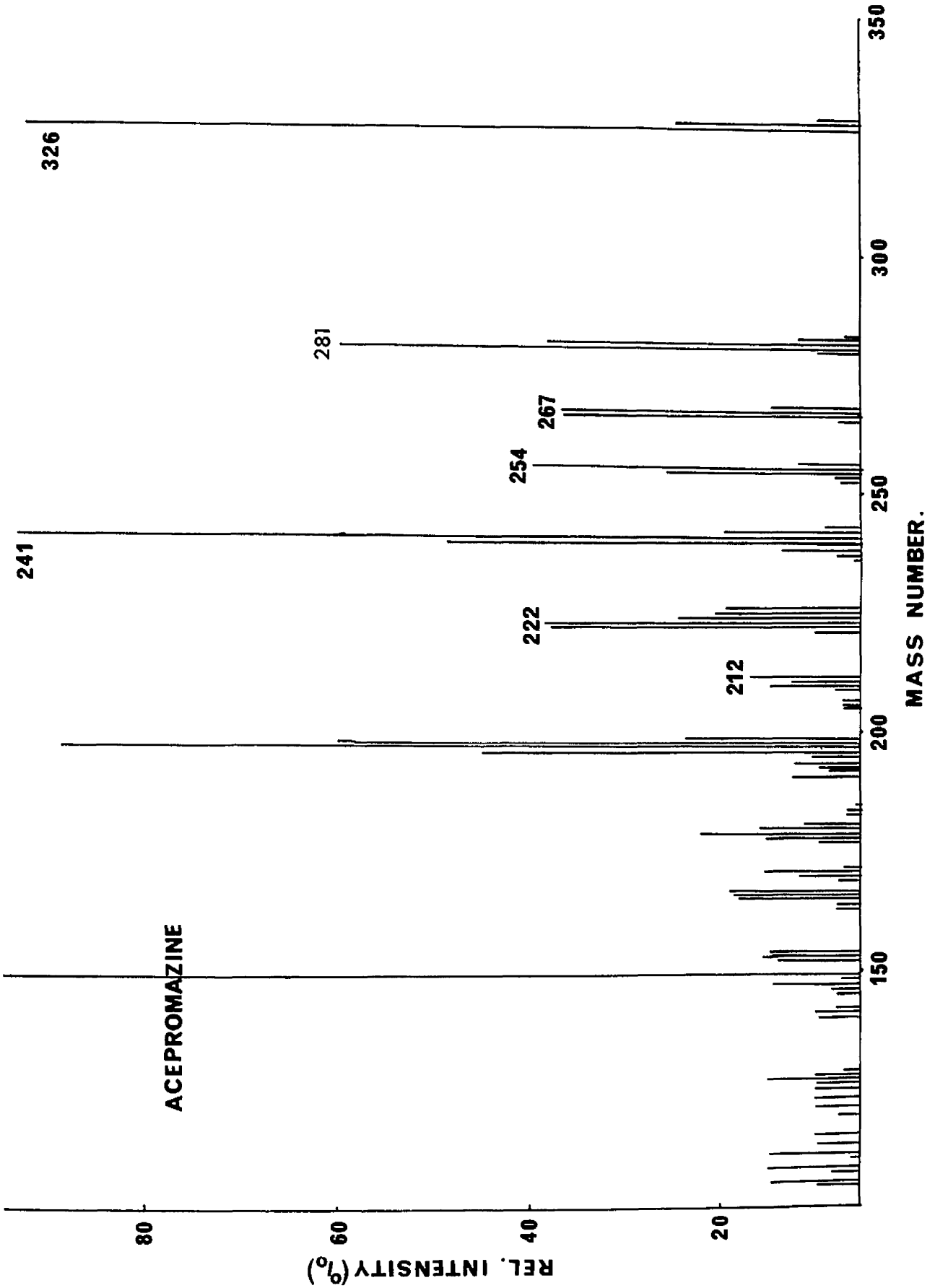
corresponded to the molecular weight of 2-chloro-phenothiazine, and by analogy with the other chloro-derivatives studied, degradation of this compound would give a derivative of mass 201. Thus although no reference compound was available, the first peak was assumed to represent 2-chloro-phenothiazine. The peak at 1.3 minutes corresponded in molecular weight and breakdown pattern to chlorpromazine. The third peak showed a molecular weight of 334 and major degradation products at mass numbers 318, 289, 273, 259, 246, 214, 199 and 167, corresponding to the spectrum of chlorpromazine sulphoxide.

Thus chlorpromazine sulphoxide is partially decomposed in the gas chromatograph-mass spectrometer to chlorpromazine and 2-chlorophenothiazine. This adds support to the conclusion that these are the major products of decomposition on metal columns (Pg. 242). Also the breakdown products of chlorpromazine and its derivatives in the mass spectrometer correspond to the breakdown series shown for promazine (Figure 59), except that the chlorine atom is not broken from the nucleus.

(c) Acepromazine.

Acepromazine and its sulphoxide were also run on the gas chromatograph-mass spectrometer. The mass spectrum of acepromazine showed a molecular weight of 326 and the figures for the major degradation products were 281, 267, 254, 241, 222 and 212. These mass numbers, with the exception of 212, correspond to those for the breakdown of promazine with 42 mass units added, (i.e. the increase in mass on addition of an acetyl group). Mass number 212 corresponds to that of a breakdown product of promazine, (compound

FIGURE 61


ACEPROMAZINE
326
281
267
254
241
222
212
REL. INTENSITY (%)
20
40
60
80
150
200
250
300
350
MASS NUMBER.

C). Thus acepromazine is degraded in the mass spectrometer in the same manner as promazine, the acetyl group remaining attached to the nucleus until fragments of mass number less than 220 are obtained, (Figure 61).

Acepromazine sulphoxide, like the other two sulphoxides, produced three chromatographic peaks. At 250°C their retention times were 1.3, 2.8 and 4.8 minutes. The peak at 1.3 minutes showed a molecular weight of 241 which would correspond to 2-acetyl phenothiazine. Thus, by analogy, with the mechanism of decomposition of the other two sulphoxides, although no reference compound was available, the first peak was identified as 2-acetyl phenothiazine. The molecular weight and breakdown pattern of the second peak was identical to that of acepromazine. The peak occurring at 4.8 minutes showed a molecular weight of 342 and had derivatives of degradation of mass 326, 297, 281, 267, 254, 241 and 222 corresponding to the mass spectrum of acepromazine sulphoxide. It was thus concluded that acepromazine sulphoxide had been thermally decomposed in the gas chromatograph-mass spectrometer to acepromazine and 2-acetyl phenothiazine.

The characteristic fingerprint pattern and clearly defined molecular weight obtained for each compound using the mass spectrometer, and the single peaks obtained for most derivatives on the gas chromatograph, suggested that this combination of instruments might be of considerable use in the structural determination of metabolites.

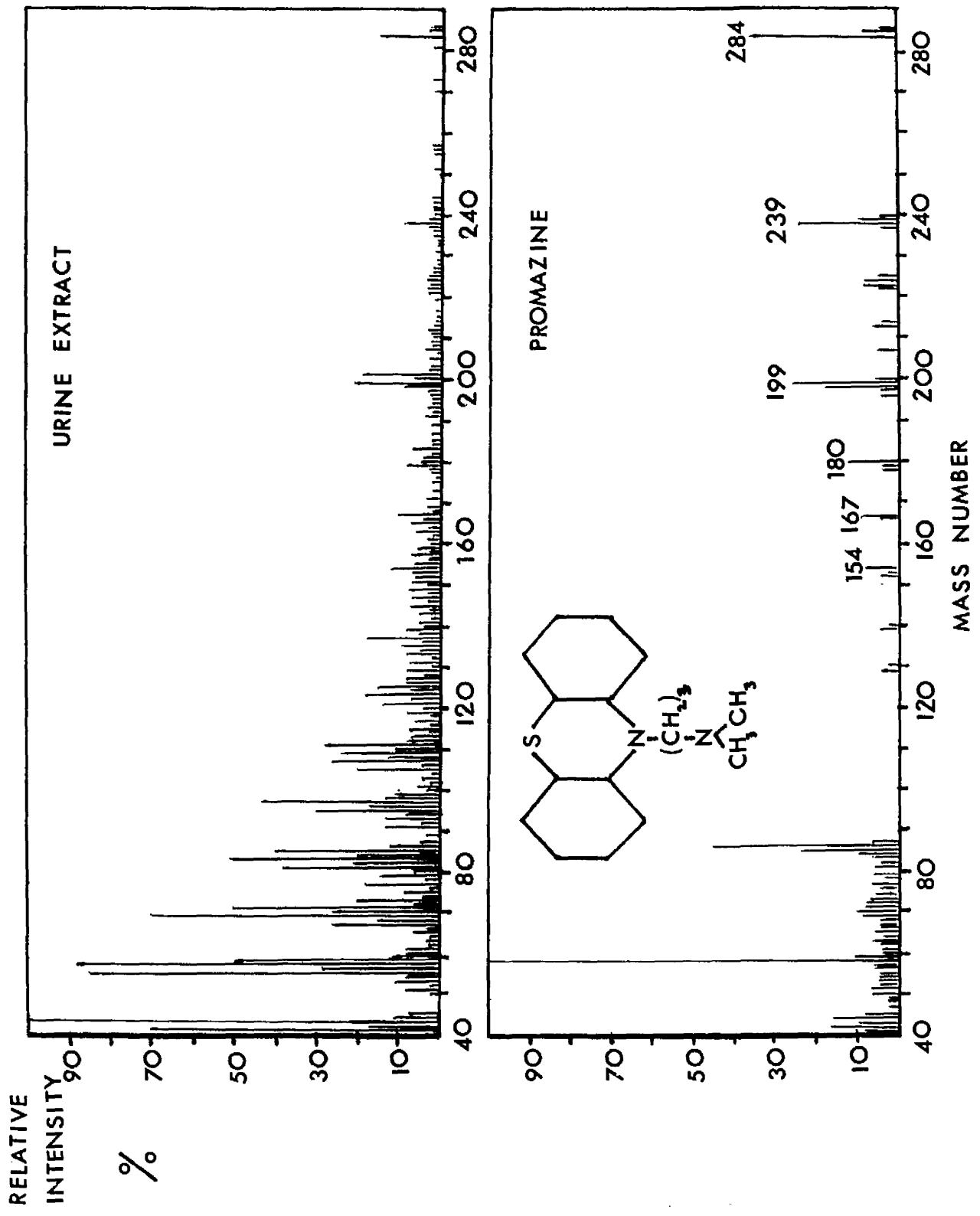
(d) Studies on Urine Extracts.

To determine the best operating parameters, urine extracts were first chromatographed on glass columns, (1% SE 30), using the gas chromatograph alone. The samples were then run on the gas chromatograph-mass spectrometer, and spectra were recorded at each peak. Preliminary experiments were carried out on "promazine" extracts, a series of increasingly concentrated samples (10:1 - 400:1) being chromatographed.

In each case a large number of peaks were recorded (over 30 for the most concentrated samples). This was especially true for "conjugated" extracts. However, on examination of their mass spectra, only one or two at the most from each extract resembled those of phenothiazine derivatives. The others, due to contaminants extracted from the urine, had molecular weights ranging from 150 to 400. They often had cracking patterns showing intermediates which could have been breakdown products of phenothiazine derivatives, suggesting that separation was not complete. However, further attempts at separation by temperature programming between 150 and 250^{o}C were unsuccessful. The parent drug (Figure 62), promazine sulphoxide and a hydroxylated derivative were occasionally detected by this method using the more concentrated extracts.

Thus, due to contaminants this technique is not so applicable to separation and identification of urinary metabolites of promazine as was at first anticipated. Similar results were obtained using chlorpromazine and acepromazine. Metabolites may be detected occasionally, but the chances are much greater that they will be

FIGURE 62


MASS SPECTRA FROM GC–MS
URINE EXTRACT
PROMAZINE
RELATIVE INTENSITY
%
90
70
50
30
10
40
80
120
160
200
240
280
154
167
180
199
239
284
MASS NUMBER
S
N
(CH2)3
N
CH3
CH3

masked by contaminants. The method would be of much greater use if extracts could be more thoroughly purified. Elution of spots from thin layer plates was attempted but no "phenothiazine" spectra were obtained, probably due to the low recoveries of such compounds from plates using organic solvents (Pg. 119).

At present the method is to some extent a matter of trial and error since so many peaks must be investigated but is of use in indicating, by cracking patterns, that "phenothiazines" have been administered.

DISCUSSION

VII DISCUSSION

Detailed studies of drug metabolism and excretion in the horse are rare. Most investigations have been aimed primarily at the detection of potential doping substances rather than at quantitative evaluation of their metabolic routes, and the methods published are not always sensitive or specific. Schubert (1967) investigated methods of detection of a series of drugs and their metabolites after administration to this species. These included salicylic acid, phenylbutazone, caffeine, amphetamine and chlorpromazine. However, the studies were mostly qualitative in nature, and the reports were not detailed. Nicholson (1968) investigated the metabolism and length of urinary excretion of phenobarbitone, pentobarbitone and their metabolites, and determined the percentages of dose excreted. He also studied the metabolism of ephedrine by the horse, but only semiquantitative results were reported (Nicholson, 1969).

Compared with the mass of information on metabolism of the phenothiazine tranquillisers by man, dogs and rats (Chapter II) little has been reported on such processes in the horse. Carey and Sanford (1963) detected the presence of 7 metabolites of promazine in horse urine, but did not identify them. Schubert detected 4 metabolites of chlorpromazine one of which was identified as the sulphoxide. He found that only a small percentage of the dose was excreted, although excretion lasted for several days.

Nearly all reports on the excretion of phenothiazine tranquillisers have been concerned with pooled 24 or 48 hour urine samples and

quantitative excretion patterns have only occasionally been investigated over smaller time intervals. Since so little information was available on this subject the present study was designed to follow metabolism and excretion of the tranquillisers more commonly used in equine practice in detail over such small time intervals. Due to the large number of biotransformations of such compounds found in other species, excretion of metabolites was followed continuously in six major groups (Pg. 141). This gave a pattern of variations in rates of excretion of individual metabolic groups over successive 8 hour intervals until excretion had reached the limits of detection of the technique. Since such compounds are usually administered to horses in a single dose, compared to the repeated daily administration to humans, and since only a very small percentage of the derivatives recovered is unchanged drug, a knowledge of such metabolic patterns is of the utmost importance in dealing with cases where administration of tranquillisers to horses is suspected.

Carr (1962) reviewed the metabolism and excretion of psychoactive drugs and summarised the complexity of such processes in the following words. "The phenothiazine derivatives that are used in the treatment of psychiatric states pose many atypical problems in psychopharmacology. Not the least of these are their unusual metabolic patterns and individual excretion differences that vary with the different drugs and different patients". The present study has shown that such processes are also complex and irregular in the horse. 30 urinary metabolites of chlorpromazine, 20 of promazine, 11 of acepromazine and 6 of propionyl-promazine have been detected. The number and amounts of individual metabolites of each drug were also found to vary considerably over

successive 6 hour intervals, which would explain the irregular excretion patterns of metabolic groups obtained by spectroscopy (Pg. 141).

Several metabolites were not fully identified due to a lack of suitable reference standards, and chemical modification, (e.g. sulphoxide formation), of those available could not be accomplished due to limited supplied (Pg.121). Thus several analytical techniques were used for structural determination including a series of specific spray reagents and elution followed by spectroscopy. Determination of the position of substitution of hydroxylated derivatives was also attempted using the spectroscopic method of Beckett et al. (1963). However, in most cases insufficient material could be eluted to give a satisfactory spectrum. This seems to be due to a combination of the small amounts excreted and strong binding of the phenothiazine derivatives to the silica gel coating of the thin layer plate.

Sulphoxide formation and hydroxylation, major metabolic routes in other species (Chapter II), were both detected. The latter was the major route for promazine and chlorpromazine and was followed by conjugation, predominantly with glucuronic acid and to a lesser extent with sulphuric acid. On the other hand, sulphoxide formation, accompanied by loss of the side chain ketone grouping at the 2-position was the major route for acepromazine and propionylpromazine.

Demethylation, which has been widely reported in other species (Ross et al., 1958; Walkenstein and Seifter, 1959; Goldenberg and Fishman, 1961; Beckett et al., 1963), did not appear to take place. However, the specific spray reagents, ninhydrin and nitroprusside, used to detect this transformation are poorly sensitive (Pg.118). Also for

preliminary experiments on standard demethylated "phenothiazines" it is clear that the success of these reactions depends to a great extent on the conditions employed, the optimum conditions varying from compound to compound. Thus on spraying standard spots of desdimethylpromazine and desdimethylchlorpromazine with the ninhydrin reagent (Pg. 118) the former produced a colour reaction within 5 minutes of heating, whereas the latter produced only a faint colouration after 10 minutes. Increasing the concentration of the ninhydrin solution did not result in a corresponding increase in the intensity of colouration. It was also noted that the nitroprusside spray (Pg. 118) did not always produce a colour reaction with desmethylchlorpromazine or desmethylpromazine. The reason for this is not apparent, but could be due to small differences in the length of time between mixing the two solutions and subsequent spraying, or to the amounts of reagent sprayed. Thus some of the metabolites detected using the more sensitive sulphuric acid reagent may be demethylated, but either due to the small amounts excreted or failure of the derivatives to react, the transformation was not detected.

Two spectroscopic techniques were used for quantitative analysis (ultra violet spectroscopy and visible spectroscopy after reaction with sulphuric acid), the latter being used to corroborate the results of the former. There was good agreement in every case. However, in preliminary experiments a build up of contaminants was noted in urine stored at $4^{o}C$ which tended to mask the absorbance of phenothiazine derivatives in extracts (Chapter V). Since the rate of formation of contaminants increased considerably on incubation of the urine at $37^{o}C$ and since their formation could be inhibited by sterilisation of the

urine, the interference was attributed to bacterial action. In addition, a build up of β-glucuronidase activity was noted in urine stored at 4°C which could also be inhibited by storage at - 20°C or sterilisation, but this did not appear to be the cause of the build up of contaminants (Pg.127). In view of these facts, storage at -20°C, thorough washing of extracts, and, in the case of conjugated metabolites, strict sterilisation (Pg.136) should be adhered to in the analysis of phenothiazine derivatives in horse urine.

Quantitative excretion patterns were variable and irregular (Pg.141). Eiduson et al. (1963) reported variable rates of excretion of thioridazine over successive short time intervals after oral administration to man. They also found that there was a correlation factor of 0.73 between amounts excreted and urinary volume, but, since they could find no such relationship after administration of chlorpromazine, they assumed this type of correlation was peculiar to thioridazine. Fisher et al. (1949) studied the urinary excretion of caffeine in several species and reported that amounts excreted were dependent on urinary volume. In the present study however, no correlation could be found between the amounts excreted or the irregular excretion patterns and urinary volume.

Several workers have reported relationships between amounts of drugs excreted and urinary pH (Weiner and Mudge, 1964). Salicylic acid is excreted at a much greater rate in alkaline urine (Smith et al., 1946; Cumming et al., 1964). The urinary excretion of amphetamine by humans is also dependent on pH, greater amounts being excreted in acidic urine (Beckett and Rowland, 1964). During studies into the metabolism of ephedrine by the horse, Nicholson (1969) noted that much greater amounts

of norephedrine were excreted in acidic urine than in alkaline urine.

In the present study the urinary pH, in the majority of cases, varied only slightly between 7 and 8.5, and the slight variations observed could neither be related to amounts excreted nor the irregular excretion patterns. Occasionally horses excreted acidic urine (pH 5.0 - 7) over a whole experiment but this was not accompanied by a corresponding increase in amounts of metabolites excreted. The occurrence of low pH values was not related to the age or sex of the animals and, since all feeds were standardised (Pg. 97), could not be related to diet. Neither could it be related to the feeding habits of the animal while at grass, since at least three days were allowed to elapse before starting any experiment, during which the animal was fed the standard diet. This time interval also allowed for a decrease in urinary volumes to much more manageable amounts (Pg.97). Racehorses have been reported to excrete acidic urine (down to pH 4.8), which was assumed to be due to the increased exercise which these animals undergo (Chapman, 1969). Thus the pH of urine collected during the present studies may be related to the activity of the animal before and during experiments.

The variable rates of excretion over successive 8 hour intervals did not appear to be related to urinary volume or pH. On the other hand, extensive localisation of phenothiazine derivatives has been reported in various organs and tissues (Salzman and Brodie, 1956; Fyodorov, 1958; Walkenstein and Seifter, 1959). Variable rates of release of metabolites from such organs (lung, liver, kidney, spleen) over successive intervals of time could give rise to the variable rates of excretion of metabolites.

In addition to the irregular excretion patterns of individual metabolic groups, two types of excretion pattern of total metabolites was

noted. In the first the rate reached a maximum within 8 hours of dosing, whereas in the second the rate rose to a much smaller maximum at approximately 24 hours after administration. Both types were noted after administration of promazine or chlorpromazine, whereas only the first type was noted after acepromazine administration. The patterns were neither related to the age nor the sex of the animals, but in duplicate experiments on the same animal, similar types of pattern were obtained. Neither was the type of pattern obtained related to the route of administration.

The animals were equally tranquillised when either pattern was recorded, and so presumably had approximately similar brain concentrations of the drug. Walkenstein and Seifter (1959), on the basis of tissue distribution studies using ^{35}S promazine, suggested that the parent phenothiazine derivatives have a lower blood-brain barrier than their metabolites and, as a result, the drugs are highly localised in the brain, whereas the metabolites are more highly concentrated in other organs (lung, liver, kidney, spleen, etc.) Thus, if brain concentrations are similar, the type of pattern obtained must depend on the concentration of circulating metabolites in the blood.

If we assume the exponential pattern the type normally to be expected there are several possible explanations for the much slower second type. Firstly, the delay in reaching a maximum rate could be due to an inability of the animal to metabolise the drug. However, very little unchanged drug was found in the urine and there was no prolongation of action when the second pattern was noted. Thus this explanation must be discounted. Another possible explanation could be poor urinary

excretion of metabolites, but volumes of urine voided and pH were similar in each type of pattern. Similarly failure of the kidneys in certain animals to excrete metabolites would be another possible explanation. On the other hand the second excretion pattern could be due to localisation of metabolites in various organs. If different animals had different capacities for binding metabolites then this would explain the two types of pattern.

The latter two explanations seem the most feasible. However, such hypotheses cannot be proved until much more information is available correlating blood concentrations, tissue distribution, metabolism, excretion and pharmacological activity of such compounds.

The percentage of dose excreted in every case was low. After oral administration 27% of the dose of chlorpromazine, 10% of promazine and 4% of acepromazine was excreted. The corresponding percentages after intramuscular administration were 10%, 11% and 1.3%. This is in agreement with work carried out in other species, although individual reports vary considerably (Emmerson and Miya, 1963). Nadeau and Sobolewski (1959) recovered 5 - 20% of daily oral doses of chlorpromazine from human urine over 48 hours. Dubost and Pascal (1953) recovered 7% of a dose of chlorpromazine from a 24 hour sample of rabbit urine. Beckett et al. (1963) also recovered 7% of an oral dose of chlorpromazine from humans over 48 hours.

The latter workers postulated the low recovery was either due to predominantly biliary excretion of metabolites, or to poor absorption of the drug from the alimentary tract, or to poor reabsorption of

metabolites excreted in the bile. However, in the present study recovery was low after parenteral as well as oral administration and thus cannot be attributed to poor absorption from the intestine. Also approximate pK values measured for the parent drugs were between 6 and 8 (Promazine hydrochloride, 7.2; chlorpromazine hydrochloride, 6.5), which would not hinder their absorption from the intestine.

Biliary excretion and poor reabsorption would lead to high concentratic of metabolites in the faces. Faecal excretion of phenothiazine metabolites has been reported by several workers (Fyodorov and Shnol, 1956; Emmerson and Miya, 1963; Eiduson and Geller, 1963). They measured radioactivity in the faeces after administration of ^{35}S labelled tranquillisers and assumed this was due to phenothiazine metabolites. However, no metabolites have been detected in faeces using spectroscopic or colorimetric techniques. Thus the activity measured in the faeces may be due to "non phenothiazine" metabolites. This would also account for the much larger percentages of dose detected in urine using labelling techniques. During the present study examination of faeces using the spectroscopic techniques described showed no evidence of phenothiazine derivatives. Thus the low percentages of dose recovered in the urine do not appear to be due to predominantly biliary excretion of metabolites.

On the other hand the extensive localisation of such compounds in different organs and tissues previously described could account for the low percentages of dose recovered. Slow release from such sites of localisation would also account for the prolonged excretion observed, which has also been noted in other species (Berti and Cima, 1956; Forrest et al, 1959; Walkenstein and Seifter, 1959; Huang et al., 1961; Emmerson and Miya, 1962).

Much larger percentages of dose were excreted after oral dosing than after intramuscular administration in the present study, especially over the first 32 hours after administration (Pg.181). Also, on the one occasion that promazine was administered intravenously the percentage of dose excreted over the first 32 hours was comparable with that after oral administration. Berti and Cima (1956) while studying the metabolism and excretion of chlorpromazine by the rabbit also noted that the percentage dose excreted was much greater after the oral route than after subcutaneous or intramuscular injection. Similar observations were reported by Burns et al. (1953) who studied the metabolism distribution and excretion of phenylbutazone in man. The latter workers believed the drug to be absorbed rapidly after oral administration but that, after the intramuscular route, precipitation or conjugation at the site of injection took place.

Fyodorov (1958) studied the metabolism, distribution and excretion of promazine, chlorpromazine and chlormepazine in rabbits, rats and dogs after different routes of administration. They found extensive localisation of the drugs at the site of injection after intramuscular, intraperitoneal and subcutaneous administration, whereas they were rapidly absorbed after the oral route. They postulated that the binding was due to a combination of inflammation of the tissue at the site of administration and alkaline hydrolysis of the salt form of the drug to the free base. They also reported that different drugs had different capacities for binding to tissues, which would explain the different percentages of dose obtained for the drugs studied in the present work.

It is thus postulated that phenothiazine derivatives are also localised in muscle tissues at the sites of injection after intra-

muscular administration to the horse, followed by slow release from these sites. On the other hand, after oral administration, such compounds are rapidly absorbed. Also the fraction of drug not bound at the sites of injection would not be so rapidly metabolised as after oral administration. The low blood-brain barrier for unchanged drug compared to its metabolites (Walkenstein and Seifter, 1959) would thus account for the similar degree of pharmacological effect noted after both routes of administration. However, due to the scarcity and value of the experimental animals employed, it was not possible to test this hypothesis by slaughter and subsequent analysis of tissues at the sites of intramuscular injection.

The conjugated fraction was assayed spectroscopically after enzymic hydrolysis of the conjugates. Enzymic incubation was employed rather than chemical hydrolysis for several reasons. Firstly the use of specific enzymes allowed analysis of individual conjugated fractions which is not possible by chemical hydrolysis. Also it has been reported that sulphoxide derivatives of such drugs are reduced to the corresponding sulphide on heating with hydrochloric acid (Schmalz and Burger, 1954). Thus this type of hydrolysis would not give a true representation of the metabolites present. Strong alkali has also been reported to decrease the intensity of ultra violet spectra of chlorpromazine (Fels, Kaufman and Karczmar, 1958), which would also produce misleading quantitative results.

Glucuronide conjugated metabolite of promazine and chlorpromazine predominated which is in agreement with findings in other species. Beckett et al. (1963), after oral administration of chlorpromazine to

man reported the ratio of glucuronide to unconjugated to sulphate conjugated metabolites as 12:5:4. Flanagan et al. (1959) found, after intraduodenal administration of chlorpromazine to dogs that the ratio of the "bound" (conjugated) to the "free" (unconjugated) fraction in the urine was approximately 3:1. Nadeau and Sobolewski (1959) reported that 50 - 98% of metabolites of levomepromazine or chlorpromazine excreted after oral administration to man were glucuronide conjugates. In the present study the ratios of glucuronide to unconjugated metabolites after intramuscular administration of promazine or chlorpromazine were 5:1 and 7:1 respectively. The corresponding ratios after oral administration were 6:1 and 18:1.

Sulphate conjugated metabolites were detected in only trace amounts after intramuscular injection but were found in much greater quantity after oral administration ($\sim$1.3% for each drug). Sulphate conjugated metabolites of chlorpromazine, accounting for approximately 1.3% of the dose were recovered from human urine by Beckett et al. (1963). Ethereal sulphates of promazine have also been reported in the urine of rabbits, rats and man. In man they accounted for approximately 3% of the dose (Goldenberg et al. 1964). However, both groups of workers studied only oral administration. Posner et al. (1963) failed to detect any sulphate conjugated metabolites of chlorpromazine in human urine after sulphatase incubation, but did not specify the route of administration. No reports of detection of sulphate conjugated metabolites after intramuscular administration of these drugs have been found.

During the present work preliminary <u>in vitro</u> studies using rat liver homogenates have shown that all three metabolic fractions examined

are formed in the liver. Since both glucuronide and sulphate conjugation occurs in the liver and since glucuronides are excreted in measurable amounts after intramuscular administration, it is postulated that a concentration barrier may exist for the formation of sulphate conjugates. If drugs were localised at the site of intramuscular administration this could prevent a high enough concentration being attained in the liver for sulphate conjugation to take place. On the other hand the required concentration would be reached after the oral route, when most of the drug absorbed passes through the portal circulation. This would explain the lack of sulphate conjugates after intramuscular administration.

There have been no previous reports on the metabolism of acepromazine or propionylpromazine in any species, which is surprising, especially in view of the widespread use of the former compound in veterinary medicine. The present work has shown that unlike the other compounds studied they are not conjugated to any great extent. It has also been reported that thioridazine does not undergo conjugation to any great extent (Eiduson and Geller, 1963) but no reasons were given. Instead of conjugation acepromazine and propionylpromazine undergo sulphoxidation and loss of the nuclear ketone grouping. A small percentage of the dose does retain the latter group, is hydroxylated and further conjugated as glucuronides or sulphates. These metabolic routes have been found to apply to both horses and dogs.

There is the possibility that sulphoxidation of phenothiazine derivatives may precede hydroxylation in the body, or that the two processes take place independently at different enzymic sites. Beckett et al. (1963) suggested that the latter mechanism operated followed by

partial rearrangement of the sulphoxide to the 3-hydroxy derivative as described by Craig and Tate (1961) (Figure 3). This would explain the presence of conjugated metabolites of promazine and chlorpromazine predominantly as sulphide derivatives as opposed to the sulphoxide forms of the unconjugated fraction. It would also explain the predominance of conjugated metabolites of these drugs over the unconjugated fraction.

In the case of acepromazine and propionylpromazine it is postulated that sulphoxide formation and hydroxylation again take place independently but that rearrangement of the sulphoxide is largely inhibited by the electron withdrawing ketone group in conjugation with the benzene ring. This would account for the much smaller proportions of conjugated metabolites of these drugs obtained. It is further postulated that the ketone grouping is subsequently cleared from the sulphoxide derivatives, but not from the hydroxylated forms giving rise to the characteristic spectra and high percentages excreted in the unconjugated fraction.

The amounts and types of metabolites excreted were neither related to the age of the sex of the animals but, in duplicate experiments using the same horse metabolism and excretion were found to proceed along similar lines. Certain abnormalities inconsistent with the expected excretion patterns were found for individual animals. Horse 4 excreted much larger percentages of dose than other animals undergoing similar experiments (Pg.182). This was attributed to a much greater capacity of this horse for formation of glucuronide conjugated metabolites. Also, although most horses excreted glucuronide conjugated metabolites predominantly in the sulphide form, horse 7 excreted this fraction almost entirely as sulphoxide derivatives on every occasion. This

seems to be a peculiarity of this horse for which there is no obvious explanation.

After administration of small doses of each drug sufficient to slow the animals in a 200 metre gallop test (Pg.224) metabolites were just on the limits of detection of the spectroscopic techniques employed. In some cases only unconjugated metabolites were detected whereas in other cases only the conjugated fraction was noted. However, using thin layer chromatography, metabolites were detected even after the lowest effective doses of acepromazine. Thus in cases where administration of phenothiazine derivatives is suspected all three metabolic fractions should be investigated by both techniques. The characteristic breakdown patterns of the phenothiazine derivatives in the mass spectrometer (Pg.246) may also prove invaluable in cases of suspected doping by these compounds.

In conclusion, the present study has borne out the complex and irregular metabolism and excretion of the phenothiazine tranquillisers experienced in other species. It has also shown the different metabolic routes of acepromazine and propionylpromazine compared to other members of the series. Since at least one of the metabolic fractions of such derivatives can be detected by the methods of analysis employed, these methods should prove of value in cases where their illegal administration is suspected.

CONCLUSIONS

The following conclusions have been reached regarding metabolism and excretion of phenothiazine tranquillisers by the horse.

(1) Excretion of individual metabolites and metabolic groups is irregular, and rates of excretion over successive time intervals can be divided into two patterns, one attaining a maximum rate almost immediately, and the other rising slowly to a maximum at approximately 24 hours after administration.

(2) Rates of excretion are not dependent on urinary volume or pH.

(3) The percentages of each dose detected as urinary metabolites are low and excretion is prolonged, lasting 6 to 7 days for promazine.

(4) Results suggest a high degree of localisation of metabolites in various tissues and organs.

(5) Unconjugated metabolites are excreted predominantly as sulphoxide derivatives whereas conjugated metabolites are mostly in the sulphide form.

(6) Glucuronide conjugates of promazine and chlorpromazine predominate, whereas acepromazine and propionylpromazine are excreted mostly in unconjugated form as sulphoxide derivatives of promazine.

(7) Sulphate conjugated metabolites are excreted in only trace amounts after intramuscular administration but are present in much greater quantity after the oral route.

(8) Individual excretion differences were noted for different horses, routes of administration and drugs.

(9) Metabolism is complex, a large number of biotransformations taking place for each drug.

(10) The methods of analysis described are sufficiently sensitive to allow detection of doping by such derivatives.

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